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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 31/70, A61P 11/06 // A61K 39/35	A1	(11) International Publication Number: WO 00/50044 (43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/US00/04780

(22) International Filing Date: 25 February 2000 (25.02.00)

(30) Priority Data:
60/121,547 25 February 1999 (25.02.99) US

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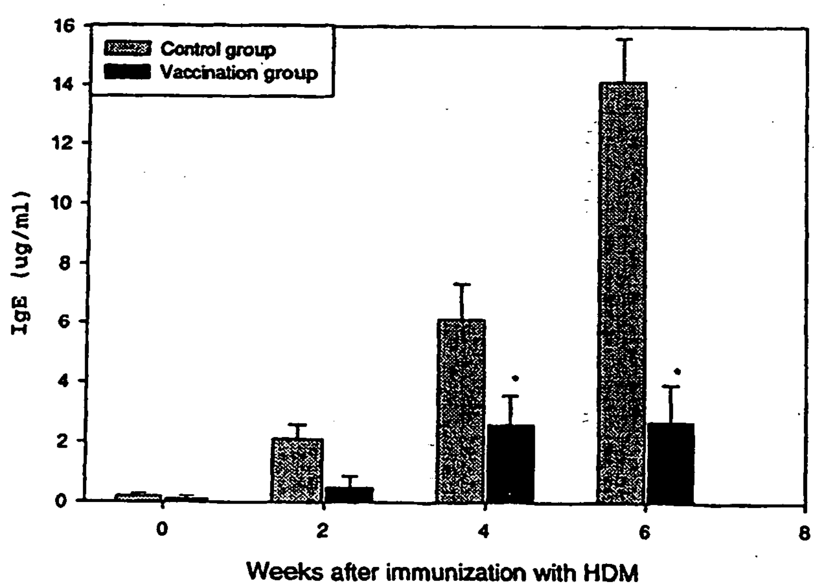
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VACCINE FOR HOUSE DUST MITE ALLERGEN USING NAKED PLASMID DNA

Total IgE levels



(57) Abstract

Vaccination with the DNA encoding T–cell epitopes to the house dust mite Dermatophagoides pteronyssimus (Der p) and Dermatophagoides farinae (Der f) were effective in the inhibition of the allergen induced IgE synthesis. Gene therapy using T–cell epitope encoding DNA is useful in combating allergic disease.

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VACCINE FOR HOUSE DUST MITE ALLERGEN USING NAKED PLASMID DNA

CROSS REFERENCE TO RELATED APPLICATION

The present application claims the benefit of co-pending U.S. Provisional Patent Application Serial No. 60/121,547 filed February 25, 1999, which is incorporated by reference in its entirety herein.

FIELD OF THE INVENTION

The present invention relates to a method of vaccination using naked DNA encoding T-cell epitopes, such as those of the house dust mite Dermatophagoides pteronyssimus (Der p) and Dermatophagoides farinae (Der f), which results in the suppression of IgE production. The present invention also relates to novel combinations of mixed plasmid DNA useful in IgE suppression.

BACKGROUND OF THE INVENTION

Genetic vaccination with naked plasmid DNA provide long standing cellular and humoral immune response and promoted a shift in the pattern of cytokines produced by the T cells. Recently peptides derived from T-cell epitopes can downregulate cytokine production and prevent specific antibody formation and administration of a single dominant epitope may tolerize the response to all the T-cell determinants within that protein.

About 15% of the world population exhibit a hypersensitivity response to common aeroallergens resulting in asthma, eczema, and rhinitis. The most frequently implicated allergens are derived from the house dust mite (HDM) including Dermatophagoides pteronyssimus (Der p) and Dermatophagoides farinae (Der f). From the serological analysis of IgE antibodies from HDM-allergic individuals a major component (>90%) of the humoral response is reactive with the group 1 and group 2 allergen. Therefore, using truncated recombinant proteins and overlapping peptides based on the nucleotide sequences, it is possible to generate T-cell epitope maps for human responses to HDM-derived allergens and to allow the development of immunotherapy. However, this vaccines using peptides has substantial limitations that have poor immunogen. Recently, it has revealed that genetic vaccinations with naked DNA provide long-lasting cellular and humoral immune responses. Long-term persistence of plasmid DNA and foreign gene expression in muscle suggested that muscle is an attractive target tissue for gene vaccination. Many studies have revealed that gene immunization with plasmid DNA encoding whole allergens or protein antigens induced strong a T helper type (Th1) immune responses in mice and rats.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method of vaccination using naked DNA encoding T-cell epitopes, such as those of the house dust mite Dermatophagoides pteronyssimus (Der p) and Dermatophagoides farinae (Der f), which results in the suppression of IgE production. The present invention also provides a novel combinations of mixed plasmid DNA useful in IgE suppression.

A composition for reducing IgE production, comprising: a pharmacologically acceptable medium and a substantially pure, immunogenic plasmid DNA encoding a T cell eptitope. The present invention provides a method of reducing reducing IgE production, comprising administering a composition comprising a pharmacologically acceptable medium and a substantially pure, immunogenic plasmid DNA encoding a T cell eptitope. The present invention also provides a vaccine for reducing the severity of an allergic disease in a mammal, comprising a pharmaceutically acceptable carrier and at least one plasmid DNA selected from a group consisting of the house dust mite Dermatophagoides pteronyssimus (Der p), Dermatophagoides farinae (Der f) and mixtures thereof. The present invention further provides a composition for reducing IgE production, comprising: a pharmacologically acceptable medium and a substantially pure, immunogenic plasmid DNA encoding a major HDM allergen selected from a group consisting of Der p 1, Der p 2, Der p 3, Der f 1, Der f 2, Der f 3 and mixtures thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The various features and advantages of the invention will be apparent from the attached drawings, in which like reference characters designate the same or similar parts throughout the figures, and in which:

Figs. 1A-H, 2A-F and 3A-C are graphs and charts of various experimental results.

Fig. 2E shows several slides from experimental results.

Figs. 4A-D show photomicrographs of various samples.

Figs. 5A-E show photomicrographs of various samples.

The present invention will be further described in connection with the following Examples, which are set forth for purposes of illustration only. Parts and percentages appearing in such Examples are by weight unless otherwise stipulated.

EXAMPLES

Example 1 – Human epitope vaccination

INTRODUCTION

To determine whether the vaccination of naked plasmid DNA coding only a T-cells epitope peptide is able to suppress the allergic reaction in vivo, the mixed naked DNA plasmids encoding the five class of human T cell epitopes on Der p 1 and Der p 2 as genetic vaccination in BALB/c mice were used. The control mice were injected with the pcDNA 3.1 blank vector. There was a reduction in the total and Der p-specific immunoglobulin E (IgE) synthesis in the vaccinated mice comparing with the control mice. In the Der p specific-IgG2a antibody response, the vaccinated mice showed more prominent responses than the control mice. Also analysis of the cytokines serum levels after immunization of Der p extract revealed that in the vaccinated mice there was an elevation in the level of interferon-γ, a Th1 cytokine associated with suppression of IgE production. The histologic studies showed that the much less infiltration of inflammatory cells were observed in lung tissue of the vaccinated mice than that of the control mice.

To evaluate whether the vaccination of naked plasmid DNA coding only a T-cell epitope peptide suppress allergic reactions as effectively as the vaccination with

DNA encoding whole allergen or not, an immune response by gene immunization with plasmid DNA encoding major T-cell epitopes in Der p 1 and 2 to challenges with whole Der p extract in mice to mimic realistic clinical setting was investigated. It was demonstrated that genetic vaccination indeed induced the strong Th1 immune responses which reduced the IgE antibody production and allergic responses against Der p. Therefore, it would be ideal to develop an alternate naked DNA vaccination method which could be even safer than injecting whole segments of the encoding region of either Der p1 or Der p2.

MATERIALS AND METHOD

Mice

20 BALB/c mice at the age of 6-8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Tennessee (Memphis, TN). This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tennessee.

Plasmid construction

Total mRNA was isolated from Der p and Der f HDM, respectively. By using murine leukemia virus reverse transcriptase and random hexanucleotide primer following the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchberg, NJ), first-strand cDNA was generated from 1 μg of total RNA and subjected to RT-PCR. The cDNA was used PCR using Taq polymerase with

primers specific for human T-cell epitopes of Der p 1 and 2. These primers cover the mature excreted region of each genes and include EcoR1 and Xb1 site for cloning and used in this study are summarized in Table 1. The amplified PCR products were subcloned into pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA) and then sequenced.

DNA preparation and vaccination

Each plasmid construct was prepared using Maxi prep (Quiagen, Chatsworth, CA). Mice were vaccinated by injection with 300 μg of pcDNA3.1 blank vector in 100 μl of PBS (control mice) or same amount of the mixed naked DNA encoding the human T-cell epitopes on Dep 1 and 2 (vaccination mice) three times at weekly intervals into muscle.

Immunization and inhalation of allergen to mice

The Der p-induced sensitivity in a mouse model was performed as described. Der p was emulsified with an equal volume of complete Freund's adjuvant (CFA) for immunization. Three weeks after last vaccination, mice were sensitized subcutaneously at the base of the tail with 100 μg of Der p extract in CFA. The mice were also given an intraperitoneal dose of 300 ng of purified pertussis toxin at 24 and 72 hours after first immunization. Seven days later, the mice were boosted again with the same amount of antigen in incomplete Freund's adjuvant. Mice were inhaled intranasal with 10 μg of Der p extract six times at weekly intervals from boost.

Determination of total and Der p-specific IgE

The bloods from the 7 mice in two groups were collected three times on week 0 (first immunization), 3,and 6. The total IgE level was determined by ELISA as follows. One hundred microliter of anti-mouse IgE capture mAb (clone R35-72; Pharmingen, San Diego, CA) were added in each well to plate and incubated overnight at 4°C. After washing, two hundred microliter of 10% fetal calf serum were incubated at room temperature for 30 min. The plates were washed five times with washing buffer and incubated with the diluted mouse serum overnight at 4°C, followed by the addition of one hundred microliter of HRP-conjugated anti-mouse IgE detection mAb (clone R35-118; Pharmingen, San Diego, CA) overnight at 4°C. The plates were washed five times before adding citric acid-phosphate buffer (pH 5.0) containing 0.15 mg/ml of O-phenylenediamine (Sigma, St. Louis, MO). The color was developed at room temperature, and the reaction was stopped by 2.5M sulfuric acid. The color was measured at 492nm(Bio-Rad, Richmond, CA). The purified mouse anti-IgE antibody (Pharmigen, San Diego, CA) was used for total IgE standard. In the measure of the Der p specific IgE, the plate were coated with 25 μg/ml Der p in 0.1 M carbonate buffer (pH 9.6) and serum samples were diluted fivefold in 10% FCS. The other procedures were same as the measurement of Der p-specific IgE. The level of Der p-specific IgE were referenced to the standard serum pooled from six mice that were immunized with 100 μg of Der p twice and inhaled with 10 μg of antigen six times. The standard serum was calculated as 100 ELISA units/ml.

Determination of Der p specific IgG, IgG1, and IgG2a

The Der P specific IgG, IgG1, and IgG2a were determined by ELISA as

follows. Purified antigens (5 μg/ml) were coated onto the assay plated overnight at 4°C. The other procedure were same as the measurement of Der p-specific IgE.

Cytokines serum levels in BALB/c mice after immunization of Der p

The bloods from the 7 mice in two groups were collected two times on week 0(first immunization), and 2 weeks. The levels of IFN-γ and IL-4 were measured using the antibody pairs purchased from PharMingen, according to the manufacture's instruction.

Histological examination of lung tissue

Mice were anesthetized with a mixture of ketalar (35mg/ml), rompun (0.6%/ml) and atropine (0.1mg/ml), of which 0.2 ml was injected intramuscularly. The vascular bed of the lungs was perfused with 0.01M Phosphate-buffered saline (PBS) and then with 4% paraformaldehyde 0.1M PBS buffers. Whole lungs were taken out and were stored in 4% paraformaldehyde for 24 h at 4°C. After fixation, these tissues were dehydrated and embedded in paraffin. Frozen sections cut at 6 μm in thickness were stained by hematoxylin and eosin. After coding, the sections were evaluated by two observers using light microscopy. The amount of mononuclear cells per section was scored using the method described by Hessel et al. This scoring method discriminates between the presence of mononuclear cells around blood vessels (score 0-3), and around bronchioli (score 0-3), and the number of patchy cellular infiltrates (score 0-3). Histological scores were analyzed using non-parametric Mann-Whitney *U* test. At least five mice were examined.

Lymph Node Cell Proliferation

The proliferation assay was performed as described. Briefly, 10 days after immunization lymph nodes were removed aseptically, and single-cell suspension was prepared. The cells (2×10^5 cells per well) were cultured with the serial dilution of Der p (range, 0.01- 10μg/ml). Cultures were set up in 200 μl RPMI1640 supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah), 1 mmol/L sodium pyruvate, 100μg/ml penicillin, 100μ/ml streptomycin, 2 mmol/L glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 20 mmol/L HEPES (pH 7.4), and 50× nonessential amino acids. After 72 hours culture, 1μCi of [^{3}H] thymidine (Du Pont, Wilmington, Del) was added to each well. Eighteen hours later, cells were harvested, and measured by liquid scintillation counting. Values were expressed in counts per minute as follows: Counts per minute with antigen-Counts per minute without antigen. Each sample was run in triplicate. RPMI medium 1640, sodium pyruvate, penicillin, streptomycin, glutamine, HEPES, and 50 × nonessential amino acids were purchased from Irvine Scientific (Santa Ana, Calif.), and 2-mercaptoethanol was purchased from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis

Data in immunoglobuline response were analyzed by Student's paired *t* test for comparisons between control and experimental mice. Histological grades were analyzed using a non-parametric Mann-Whitney *U* test. Data were expressed as mean±SD. A *P* value< 0.05 was considered significant.

RESULTS

Suppression of total and Der p-specific IgE antibody production by gene vaccination.

To effect of vaccination with DNA encoding T cell epitopes, we examined total and Der p specific -IgE antibodies productions by ELISA (Fig. 1). The gene vaccination with the human T cell epitopes on Der p 1 and 2 showed about 50% inhibition of Der p-specific IgE and more than 50% inhibition of total IgE as compared with the control mice at week 6. Thus, genetic vaccination could inhibit an in vivo allergen-specific IgE synthesis efficiently. To study the effects of DNA vaccination on B cell immunity, we measured Der p specific serum antibodies. The production of Der p-specific IgG2a antibodies in the vaccination mice were more increased than that in the control mice after 3 weeks even if Der p specific IgG responses were similar between two groups (Fig 2A,B). But in the Der p-specific IgG1 response, control mice showed more prominent production than vaccination mice (Fig 2C).

IFN-γ and IL-4 serum levels in BALB/c mice after immunization of Der p extract

To determine whether the Th1 or Th2 cytokine involve in the genetic vaccination with the human T-cell epitopes gene, we measured IL-4 and IFN-γ serum levels. The IFN-γ serum level in vaccination mice (648.29±166.78pg/ml) were observed higher than in control mice (undetectable) at 2 weeks after immunization of Der p extract (See Fig. 3A). In parallel, the IL-4 serum level were detected contrary to the result of IFN-γ (control mice 23.63±3.66pg/ml versus vaccination mice undetectable) (see Fig. 3B). Our result suggested that the genetic immunization with the plasmid DNA encoding T cell epitopes might also induce a Th2 to

Th2 cytokine shift.

Lymph Node Cells Proliferation

To determine whether the protective effect of gene vaccination was due to the deletion of T cells or the induction of unresponsiveness, we examined the lymph node cells proliferation in response to different concentration of Der p. As seen in Figure 4, lymph node cells from the vaccinated mice showed a linear stimulation when concentration of Der p ranged from 0.01 μg/ml to 10 μg/ml as well as lymph node cells from the control mice.

Histological examination of lung tissue

To examine whether the genetic vaccination effect on the cellular response of lung or not, we stained the lung at the end of the experiment by histological method. The lung from the control mice showed the much more number of mononuclear cells infiltrates around bronchioli (mean number 1.5667±0.89 respectively, versus 0.5333±0.6756 in vaccination mice), around blood vessels (mean number 0.8833±0.8847 respectively, versus 0.3833±0.6662 in vaccination mice) than vaccination mice. Also control mice were observed significantly more patch cellular infiltrates than vaccination mice (mean number 1.3±1.0939 respectively, versus 0.35±0.6331 in vaccination mice).

DISCUSSION

Diseases such as allergic asthma and rhinitis, and atopic dermatitis are all characterized by elevated levels of serum IgE. Total and specific IgE positivity also showed a close relationship with clinical symptom in atopic alllergy. The recent analyzing the antigen specificity of T-cell clones reactive with Der p 1 and Der p 2, with truncated recombinant

proteins and synthetic peptides, has allowed several sites of T-cell recognition to be identified at different locations with Der p 1 and Der p2. Several laboratories are now investigating the development of a new generation of immunotherapeutic strategies based on the modulation of T-cell function. Immunotherapy treatment has proven to be effective treating some forms of allergy, but it was poor immunogen and was needed higher than the amount derived intracelluarly from processed antigens. Recently gene immunization with naked DNA suppressed induction of IgE synthesis. These data suggest that pDNA immunization with containing the gene for the minor HDM allergen Der p 5 may induce Th1 immune responses to the encoded antigens. The Der p 5 allergen react with about 40% allergic sera but the Der p 1 and 2 allergens react with about 80% of allergic sera [19,20)]. So we tried to examine if plasmid DNA encoding for T-cell epitopes on human Der p 1 and Der p 2 would also be able to induce abrogation of allergic responses in mice when they are given the naked DNA vaccine. We have analyzed the effects of gene vaccination with plasmid encoding T-cell epitopes on Der p 1 (residue 45-67, and 94-143) and Der p 2 (residue 11-40, 61-104, and 111-129). Our results showed above 50% inhibition of total IgE and Der p-specific IgE at week (end of the study) (Fig 1.). Thus, this result suggested that gene vaccination with plasmid DNA encoding the T-cell epitopes could also suppress induction of IgE synthesis.

To determine whether the suppressive effect of gene vaccination was due to the deletion of T cells or the induction of unresponsiveness, we examined the lymph nodes cells proliferation in response to different concentration of Der p. We found that lymph nodes cells taken from BALB/c mice were able to respond by proliferation to depend on the Der p concentration, with a similar pattern to that of the cells taken from the control mice. T cell deletion has mainly been observed after administration of high doses of antigen or peptide. Furthermore, numerous experimental systems have shown that presentation of antigen by

nonprofessional antigen presenting cells (APCs) that lack co-stimulatory capacity results in anergy rather than priming [23)]. But professional APCs, Langerhans cells or macrophage, may act as APC for intramuscular DNA vaccination. Also our result has shown T cell good responses to depending concentration of Der p. Thus, gene vaccination did not induce T cell deletion or anergy. The T helper 2 (Th2) cells mainly produce IL-4, IL-5 and IL-10 that induce antibody production in B cells, above all, formation of IgE as playing a central role in allergic responses. IFN-γ is the Th1 cytokine responsible for the inhibition of IL-4-mediated IgE responses and promotes the formation of IgG2a. Protein immunization induced a Th2 response, as shown by IgG1 and IgE antibody formation and IL-4 & IL-5-secreting T cells. In contrast, gene immunization with plasmid DNA induced a Th1 response with IgG2a antibody production and IFN-γ secreting T cells. To study the immune mechanism s involved in suppression of IgE synthesis after DNA vaccination, we measured the IFN-γ and IL-4 serum levels in BALB/c mice after immunization of Der p extract. In the vaccinated mice there was an elevation in the Th 1 cytokine IFN-γ associated with suppression of IgE synthesis. In parallel, there was measured a reduction in the Th2 cytokine IL-4. Lee et al reported that the Th1 response dominated over the Th2 response and downregulated preexiting IgE antibody formation after genetic immunization. Our result showed that the genetic immunization with the plasmid DNA encoding only T cell epitopes might also induce Th2 to Th1 cytokine shift.

To study the effects of DNA vaccination on B cell immunity, we measured Der p specific serum antibodies. At 6 weeks after immunization, total serum levels of IgG2a Der p specific antibody were increased and Der p specific IgG1 response was reduced in the vaccination mice compared with control mice even if Der p specific IgG responses were similar between two groups. IgG2a is dependent on interferon-γ (IFN-γ) as an IgM-to-IgG2a

switch factor and is believed to typical for a Th1 response. In contrast, IgG1 depends on IL-4 secreted by Th2 cells [28)]. The Der p specific IgG isotype data further strengthened DNA vaccination inducing a Th1 to Th2 cytokine shift after the genetic vaccination with plasmid DNA encoding the T-cell epitopes, since the IgG2a levels in vaccination group compared with control group were enhanced antibody formation. Our result suggested that genetic immunization might suppress IgE production by the inducing a Th2 to Th1 cytokine shift.

Allergic asthma is characterized as a chronic inflammatory disease of the bronchi and it is well established that a variety of cells including mast cells, eosionphils and lymphocytes play a role in this process. After inhalation challenge, the inflammatory cells migrate from the peripheral blood to the site of inflammation in the bronchial mucosa and brochoalveolar fluid shows dominant Th2-type cytokines. Our histological evaluation revealed that significant number of patch mononuclear cell infiltrates were observed around bronchioles and blood vessels in the vaccination mice comparing with the control mice. T lymphocytes have been suggested to play a key role in orchestrating the interaction of the participating cells since they are able to release an array of cytokines which can attract, prime and activate other cell types. A successful outcome of immunotherapy has been associated with the development of suppressor T cells, which can downregulate the allergic response. It suggested that the change in the function of T cells might effect on the reduction of the inflammatory cells infiltrates of lung tissue. This data indicated that gene immunization affects not only humoral immune responses but also cellular responses.

The vaccination with mixed naked DNA encoding only T-cell epitopes might induce abrogation of allergic response in mice as effectively as DNA encoding whole segment allergen. Thus gene therapy using DNA encoding T-cell epitopes could be an ideal way of combating allergic disease in future.

Table 1

Sequence of Primers for Human T-cell epitopes of Der p1 and 2.

Der p1 epitope (residue45-67)	5'-CCG GAA TTC GCC GCC ACC ATG TCA GCT TAT TTG GCT TAC CGT-3' [SEQ.ID.NO:45] 5'-TGC TCT AGA TTG GAA GCA CAA TCG ACT AAT TC-3' [SEQ.ID.NO:46]
Der p1 epitope(residue94-143)	5'-CCG GAA TTC GCC GCC ACC ATG TAT CGA TAC GTT GCA CGA GA-3'[SEQ.ID.NO:47] 5'-TGCTCT AGA TTG CCA ATA ATGACG GCA A-3' [SEQ.ID.NO:48]
Der p2 epitope(residue11-40)	5' CCG GAA TTC GCC GCC ACC ATG CAT GAA ATC AAA AAA AGT TTT GGT -3'[SEQ.ID.NO:49] 5'-TGC TCT AGA TTA ACG GCT TCA ATT GGA ATT -3' [SEQ.ID.NO:50]
Der p 2 epitope(residue61-104)	5'-CCG GAA TTC GCC GCC ACC ATG TTA GAA GTT GAT GTTCCCGGT-3' [SEQ.ID.NO:51] 5'-TGC TCT AGA TTA ACA TTT TCA GAT TTT GGT-3' [SEQ.ID.NO:52]
Der p2 epitope(residue 111-129)	5'-CCG GAA TTC GCC GCC ACC ATG GGT GAT GAT GGT GTT TGG CCT-3' [SEQ.ID.NO:53] 5'-TGC TCT AGA TTA ATC GCG GAT TTT AGC ATG AGT AGC-3' [SEQ.ID.NO:54]

Table 2

Inflammatory cells in the lung tissue after immunization with Der p.

Group	Around bronchioli	Around blood vessels	Patch cellular infiltrates
Control	1.5667±0.89	0.8833±0.8847	1.3±1.0939
Vaccination	0.5333±0.6756*	0.3833±0.6662*	0.35±0.6331*

* $P < 0.05$ compared with the control group

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Example 2 - The effect of vaccination with DNA encoding murine T-cell epitopes on the Der p 1 & 2 induced immunoglobulin E synthesis.

We would like to examine the effect of vaccination with DNA encoding only the murine T-cell epitopes on the IgE production. Our results suggested that genetic vaccination indeed induced the Th1 cytokine immune responses which reduced the IgE antibody production and allergic responses against Der p. Therefor it would be

ideal to develop an alternative naked DNA vaccination method which could be even safer than injecting whole segments of the encoding region of Der p 1 or Der p2.

MATERIAL AND METHOD

Mice

20 BALB/c mice at the age of 6-8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Tennessee (Memphis, TN). This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animal Welfare Act (7 U.S.C.et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tennessee.

Plasmid Construction

Total mRNA was isolated from Der P and Der f HDM, respectively. By using murine leukemia virus reverse transcriptase and random hexanucleotide primer following the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchberg, NJ), first-strand cDNA was generated from 1μ g of total RNA and subjected to RT-PCR. The cDNA was used PCR using Taq polymerase with primers specific for Der p 1 & 2 epitopes. These primers cover the mature excreted region of each genes and include EcoR1 and Xb1 site for cloning and used in this study are summarized in Table 1. The amplified PCR products were subcloned into pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA) and then sequenced.

DNA Preparation and Vaccination

Each plasmid construct was prepared using Maxi prep (Quiagen, Chatsworth, CA). Mice were vaccinated by injection with 300 μg of pcDNA3.1 blank vector in 100 μl of PBS (control group) or same amount of the mixed naked DNA encoding the murine T cell epitopes on Der p 1 and 2 (vaccination group) three times at weekly intervals into muscle.

Immunization and Inhalation of Allergen to Mice

The Der p-induced sensitivity in a mouse mode was performed as described. Der p was emulsified with an equal volume of complete Freund's adjuvant (CFA) for immunization. Three weeks after last vaccination, mice were sensitized subcutaneusly at the base of the tail with 100 μg of Der p extract in CFA. The mice were also given an intraperitoneal dose of 300 ng of purified pertussis toxin at 24 and 72 hours after first immunization. Seven days later, the mice were boosted again with the same amount of antigen in incomplete Freund's adjuvant. Mice were inhaled intranasal with 10 μg of Der p extract six times at weekly intervals from boost.

Determination of Der p Specific IgG1, IgG2a, and IgE

The bloods from the 6 mice in two groups were collected six times on week 0(first immunization), 3,and 6. The Der P specific IgG, IgG1, and IgG2a were determined by ELISA as follows. Fifty microliter of Der p (5 μg/ml in 0.1 M carbonate buffer, pH 9.6) were dispensed in each well of a polystyrene microtiter plate (Cost, Cambridge, MA) and incubated overnight at 4°C. The antigen-coated

plates were washed three times in 0.05% PBS-Tween 20 buffer (washing buffer) and incubated with mice sera overnight at 4°C. The plates were washed five times with washing buffer and incubated with peroxidase conjugated anti-mouse IgG, IgG1, and IgG2a antibody (Sigma, St. Louis, MO) overnight at 4°C. The plates were washed five times before adding citric acid-phosphate buffer (pH 5.0) containing 0.15 mg/ml of O-phenylenediamine (Sigma, St.Louis, MO). The color was developed at room temperature, and the reaction was stopped by 2.5M sulfuric acid. The color was measured at 492nm (Bio-Rad, Richmond, CA). In the measure of the Der p specific IgE, the plate were coated with 25 μg/ml HDM in 0.1 M carbonate buffer (pH 9.6) and serum samples were diluted fivefold in 10% FCS. The other procedures were same as the measurement of Der p-specific IgG except HRP-conjugated anti-mouse IgE detection mAb (clone R35-118; Pharmingen, San Diego, CA). The level of Der p-specific IgE were referenced to the standard serum pooled from six mice that were immunized with 100 μg of HDM twice and inhaled with 10 μg of antigen six times. The standard serum was calculated as 100 ELISA units/ml.

Histological Examination of Lung tissue

Mice were anesthetized with a mixture of ketalar (35mg/ml), rompun (0.6%/ml) and atropine (0.1mg/ml), of which 0.2 ml was injected intramuscularly. The vascular bed of the lungs was perfused with 0.01M Phosphate-buffered saline (PBS) and then with 4% paraformaldehyde 0.1M PBS buffers. Whole lungs were taken out and were stored in 4% paraformaldehyde for 24 h at 4°C. After fixation, these tissues were dehydrated and embedded in paraffin. Frozen sections cut at 5 μm in thickness were stained by hematoxylin and eosin. After coding, the sections were

evaluated by two observers using light microscopy. The amount of mononuclear cells per section was scored using the method described by Hessel et al. This scoring method discriminates between the presence of mononuclear cells around blood vessels (score 0-3), and around bronchioli (score 0-3), and the number of patchy cellular infiltrates (score 0-3). Histological scores were analyzed using non-parametric Mann-Whitney *U* test. At least five mice were examined.

Immunohistochemical Staining for CD4+ and CD8+ T cells in Lung.

The lung tissues from the experimental and control group mice were removed after the final intranasal inhalation. The tissues were fixed with periodat-lysine-paraformaldehyde solution for 24 h at 4°C. Frozen sections cut at 4 to 6 μm in thickness were rehydrated and rinsed in cold PBS. The endogenous pseudoperoxidase was blocked with absolute methanol containing 0.5% hydrogen peroxide for 20 min at room temperature. The sections were treated with 10% normal goat serum in PBS to reduce the nonspecific binding. Biotin conjugated rat anti-mouse CD8 or CD4 monoclonal antibody (Pharmingen, San Diego, CA), diluted to 1:200 in PBS containing 0.5% bovine serum albumin, was applied to the sections and incubated overnight at 4°C. After rinsing, the sections were incubated with avidin-biotin peroxidase complexes (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature and rinsed sufficiently with PBS. The reaction was developed with 0.02% 3,3'-diaminobenzidine in 0.05 M of Tris buffer (pH 7.6) with 0.005% hydrogen peroxide for 7 min. The sections were dehydrated, cleared in xylene, and mounted.

Measuring Cytokine mRNA Expression

Mice from two groups were sacrificed 10 days after immunization with Der p extract. The lymph nodes were removed from mice and stimulated with recombinant Der p (100 μg/ml) in vitro for 18 h. the cells were washed with PBS buffer and mRNAs prepared (Biotecx, Houston, TX). By using murine leukemia virus reverse transcriptase and random hexanucleotide primer following the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchber, NJ), first-strand cDNA was generated from 1 μg of total RNA and subjected to RT-PCR analysis. To determine the relative abundance of each cytokine mRNA expression, the amount of each cDNA for PCR were optimized by the intensity of the amplified DNA products of β-actin from each RNA. In the PCR reaction mixture, either β-actin as control primer, IL-2, IFN-γ (Clonetech, PaloAlto, CA), IL-4, and IL-5 at the final concentration of 0.2 μM were added. The PCR condition was as follows: 200 μM of dNTP, 10 μCi [32P] dCTP, 50 μM Tris. HCl (pH 9.0), 50 μM NaCl, 2 μM MgCl2, 0.5 mM DTT, and two units of Taq polymerase (Perkin Elmer, Branchberg, NJ) at a final volume of 20 μl. A negative control reaction was run with each sample to verify that no PCR bands appeared in the absence of template. The optimal amplification conditions were as follows: 45s at 94°C for denaturation, 45s at 67°C for annealing, and 1 min at 72°C for elongation and the PCR cycles were 30. The amplified DNAs of β-actin, IFN-γ, IL-2, IL-4, and IL-5 had sizes of 540, 365, 413, 354, and 349 base pair, respectively. The gel was dried on whatman 3M paper and exposed to Kodak XAR film. In each electrophoresis run, intra-and inter-gel staining homogeneity was confirmed by staining intensity of molecular weight markers at both ends of the gels.

In general, amplification kinetics was monitored for each PCR run by examining aliquots of the products on the gel. Amounts of the PCR products were compared during the cycle where the amplification did not reach saturation.

Statistical Analysis

Data in immunoglobulin response were analyzed by Student's paired t test for comparisons between control and experimental group. Histological grades were analyzed using a non-parametric Mann-Whiney U test. Data were expressed as mean ± SD. A P value< 0.05 was considered significant.

RESULTS

Der p-Specific IgE, IgG1, IgG2a, and IgG Antibody Responses by Gene Vaccination.

To examine the immune response, we checked the IgG, IgG1, G2a, and IgE antibodies productions by ELISA (Figure 1,2.). The gene vaccination with the murine T cell epitope on Der p 1 and 2 showed about 60% inhibition of Der p-specific IgE as compared with the control group at week 6 (Figure 1). Thus, genetic vaccination could inhibit an in vivo allergen-specific IgE synthesis efficiently. The production of Der p-specific IgG2a antibodies in the vaccination group were more increased than that in the control group after 6 weeks (Figure 2a). But in the Der p-specific IgG1, and IgG antibodies responses, the two groups did not show any difference (Figure 2b,c).

Cytokine Gene Expression by Antigen Stimulation in Vivo

To determine whether the Th1 or Th2 cytokine involve in the genetic vaccination, we performed a RT-PCR analysis on the total RNA samples extracted from lymph node cells (1×10^7 cells per well) that were cultured in the presence of Der p (100μg/ml) in vitro for 18h. A higher mRNA expression of IFN-γ in the vaccination group was detected compared with the control group. But the level of mRNA expression of IL-2, 4 & 5 in the vaccination group were detected no different level in comparing with the control group (Figure 3). These data indicate that the vaccinations with Der p epitope DNA induced a dominant Th1 cytokine (IFN-γ) gene expression in lymph node.

Histological and Immunohistochemical Examination of Lung Tissue

To examine whether the genetic vaccination effect on the cellular response of lung or not, we stained the lung at the end of the experiment by histological and immunohistochemical method. The lung from the control group showed the much more infiltration of inflammatory cells around bronchioli, blood vessels, interstitium (table 3, Figure 4). In the immunohistochemical stain for CD4+ & CD8+ T cells showed that the more CD8+T-cells infiltrated in the submucosa and mucosa of the lung from the vaccination group (94.5 ± 6.75/mm) as compared with the control group (49 ± 4.966/mm) (Figure 5). But in the stain for the CD4+ T cells showed no difference the two group (vaccination group 98.5 ± 13.44/mm versus control group 114 ± 11.31/mm). So the results suggested that the genetic vaccination also effect on the cellular response and the CD8+ T cells of the vaccination were capable of protection against a subsequent allergenic challenge.

DISCUSSION

Diseases such as allergic asthma and rhinitis, and atopic dermatitis are all characterized by elevated levels of serum IgE. Total and Specific IgE positivity also showed a close relationship with clinical symptom in atopic alllergy. A variety of approaches targeting the suppression of IgE have been proposed as synthetic peptides as T-cell vaccine. However, synthetic peptides were poor immunogen and were needed higher than the amount derived intracelluarly from processed antigens. Recently Hsu et al.showed that gene immunization of rats with plasmid encoding Der p 5 prevent induction of IgE synthesis. These data suggest that pDNA immunization with containing the gene for the minor HDM allergen Der p 5 may induce Th1

immune responses to the encoded antigens. The Der p 5 allergen react with about 40% allergic sera but the Der p 1 and 2 allergens react with about 80% of allergic sera.[3,5)] We have analyzed the effects of gene vaccination with plasmid DNA encoding only the murine T-cell epitopes in allergic responses to whole Der p extract. Our results showed about 70% inhibition of Der p-specific IgE at 6week after immunization with Der p (Figure 1).

Animal models have established that Th2 responses are mediated by T helper cells that secret cytokines such as IL-4, and IL-5 that induce antibody production in B cells, above all, formation of IgE as playing a central role in allergic responses.[19,20] IFN-γ is the Th1 cytokine responsibility for the inhibition of IL-4-mediated IgE responses and promotes the formation of IgG2a.[21] Previous report showed that protein immunization induced a Th2 response, as shown by IgG1 and IgE antibody formation and IL-4 & IL-5-secreting T cells. In contrast, gene immunization with plasmid DNA induced a TH1 response with IgG2a antibody production and IFN-γ secreting T cells. Our data showed that the mRNA expression of IFN-γ in lymph node from plasmid DNA encoding the murine T-cell epitopes on Der p1, and 2 increased more than that from control group associated with suppression of IgE synthesis. In parallel, in a Th2 response, there was no difference between two groups. Our results suggested that the genetic immunization might induce Th1 immune response to the encoded antigen or allergen. After genetic immunization, the Th1 response dominated over the Th2 response and suppressed preexisting IgE antibody formation.

Gamma interferon promotes the formation of IgG2a and inhibits IgE production[13] even if the mechanism through which IFN-γ exerts its stimulatory effects

on IgG2a production has not been established. Our data also showed that the production of IgG2a -anti-Der p antibody was higher in vaccination group than control group. Our results supported suggest that genetic immunization might suppress IgE production by the inducing the Th1 response from T helper cells.

Allergic asthma is characterized as a chronic inflammatory disease of the bronchi and it is well established that a variety of cells including mast cells, eosionphils and lymphocytes play a role in this process. After an inhalation challenge, the inflammatory cells migrate from the peripheral blood to the site of inflammation in the bronchial mucosa and brochoalveolar fluid shows dominant Th2-type cytokines. Our histological study showed that gene vaccination induced the reduction of infiltration of inflammatory cells in lung tissues (Figure 4). It suggested that the change in the function of T cells might effect on the reduction of the inflammatory cells in lung tissue. So these results suggested that genetic immunization effected not only humoral immune responses but also cellular responses.

T lymphocytes have been suggested to play a key role in orchestrating the interaction of the participating cells since they are able to release an array of cytokines which can attract, prime and activate other cell types. A successful outcome of immunotherapy has been associated with the development of suppressor T cells, which can downregulate the allergic response. Recent report has also revealed that functionally distinct subsets of CD8+ T cells may play an important regulatory role in IgE production. But Manickan et al showed that the mechanism of genetic immunization was principally by CD4+ T cells, but not by CD8+ T cells. Our immuno-histochemical study showed more CD8+ T cells were detected in the lung of the vaccination group than that of control group (Figure 5). Peptides derived from

cytosolic proteins are generally presented to CD8+ T cells by major histocompatibility complex (MHC) class I molecules which are expressed on virtually all somatic cells. So it suggested that such endogenous production of an allergenic protein might be a useful means to induce regulatory CD8+ T cells capable of conferring protection against a subsequent allergenic challenge.

The vaccination with mixed naked DNA encoding only T-cell epitopes might induce abrogation of allergic response in mice as effectively as DNA encoding whole segment allergen. Thus genetic vaccination using DNA encoding T-cell epitopes could be ideal way of combating allergic disease in future.

Table 1

Sequence of Primers for the murine Der p1 and 2 epitopes

Der p1 epitope (residue 21-49)	5'-CCG GAA TTC GCC GCC ACC ATG ACT GTC ACT CCC ATT CGT ATG C-3' [SEQ.ID.NO:1] 5'-TGC TCT AGA TTA AGC CAA ATA AGC TGA TTC AGT TGC-3' [SEQ.ID.NO:2]
Der p1 epitope (residue 78-100)	5'-CCG GAA TTC GCC GCC ACC ATG CGT GGT ATT-3'[SEQ.ID.NO:3] 5'-GAA TAC ATC CAA CAT TGC TCT AGA TTA TTC TCG TGC AAC GTA TCG ATA GTA-3' [SEQ.ID.NO:4]
Der p1 epitope (residue 110-131)	5'-CCG GAA TTC GCC GCC ACC ATG CGT TTC GGT ATC TCA AAC TAT TGC-3' [SEQ.ID.NO:5] 5'-TGC TCT AGA TTA CAA AGC TTC ACG AAT TTT GTT TGC-3'[SEQ.ID.NO:6]
Der p 2 epitope (residue 11-35)	5'-CCG GAA TTC GCC GCC ACC ATG CAT GAA ATC-3'[SEQ.ID.NO:7] 5'-AAA AAA GTT TTG GTA TGC TCT AGA TTA GAA TGG TTT ACC ACG ATG AAT GAT-3'[SEQ.ID.NO:8]
Der p2 epitope (residue 87-129)	5'-CCG GAA TTC GCC GCC ACC ATG GAT ATT AAA-3' [SEQ.ID.NO:9] 5'-TAT ACA TGG AAT GTT CCG A TGC TCT AGA TTA ATC GCG GAT TTT AGC ATG AGT AGC-3'[SEQ.ID.NO:10]

Table 2

Oligonucleotides used for cytokine mRNA expression

Molecules	Primer Sequence (5' to 3')	Product Size
β-actin	5'-GTG GGC CGC TCT AGG CAC CAA CTC TTT GAT GTC ACG CAC GAT TTC-3'[SEQ.ID.NO:6]	540bp
IL-2	5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG GACAGAAGGCTATCCATCTCCTCAGAAAGTCC-3'[SEQ.ID.NO:7]	413bp
IFN-γ,	5'-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC TGGACCTGTGGGTTGTTGACCTCAAACTTGGC-3'[SEQ.ID.NO:8]	365bp
IL-4	5'-CAG CTA GTT GTC ATC CTG CTC TTC GTG ATG TGG ACT TGG ACT CAT TCA TGG-3'[SEQ.ID.NO:9]	357bp
IL-5	5'-TGT CTG GGC CAC TGC CAT GGA GAT TC CCA TTG CCC ACT CTG TAC TCA TCA CAC-3'[SEQ.ID.NO:10]	424bp

Table 3

Inflammatory cells in the lung tissue after immunization with Der p

Group	Around Bronchioli	Around blood vessels	Patch Cellular Infiltration
Control	1.5616 ± 0.7262	1.6438 ± 1.2733	1.6471 ± 0.4926
Vaccination	0.6835 ± 0.4947*	0.3924 ± 0.5168*	0.3333 ± 0.4815*

* P< 0.05 compared with the control group

Figure 1. Effect of vaccination on the allergen induced immumoglobulin E prodution. The bloods from the 6 mice in two groups were collected three times on week 0(first immunization), 3,and 6. The gene vaccination with the murine T cell epitopes on Der p 1 and 2 showed about 70% inhibition of Der p-specific IgE as compared with the control mice at week 6. Data shown are mean ± S.D.(n=6 per group). *P < 0.05 compared with the control mice.

Figure 2. The IgG2a (A), IgG1(B), and IgG (C) antibody responses of BALB/c mice after immunizing with Der p extract. The production of Der p-specific IgG2a antibodies in the vaccination mice were more increased than that in the control mice after 3 weeks (Figure 2A). But in the Der p-specific IgG1 & IgG response, there was no difference between two groups (Figure 2B,C). Data shown are mean ± S.D.(n=6 per group). *$P < 0.05$ compared with the control mice.

Figure 3. Cytokines gene expression. T cells were collected from the lymph nodes of control or vaccination mice 10 days post boost and cultured in the presence of no antigen, and Der p extract (100 μg/ml) for 18 h. The total RNA was extracted using TRIzol reagent and RT-PCR reactions were doing using cDNA with different primers specific for β-actin, IL-2, 4, 5, and interferon-γ. 1) Lymph node cells from control mice were cultured without Der p, 2) in the presence of Der p (100 μg/ml) 3) Lymph node cells from vaccinated mice were cultured without Der p, 4) in the presence of Der p (100 μg/ml). A higher mRNA expression of IFN-γ in the vaccination group was detected compared with the control group.

Figure 4. Histopathologic examination of lung. Lungs from control and experimental groups of mice were removed on day 45 after immunization. (A). Lung from control mouse (×100). (B). Lung from control mouse (×400). (C). lung from vaccination mouse (×100). (D). Vaccination mice showed much less the infiltration of inflammatory cells than control mice.

Figure 5. Immunohistochemical examination of lung. Lungs from control and vaccination group were removed on day 45 after immunization and were stained for CD8+ T cells. (A). Lung from control mouse (×100). (B). Lung from vaccination mouse (×100). The more CD8+ T cells were observed in vaccination mice in comparing with control mice.

Example 3 - Suppressive effect on the allergen-induced immunoglobulin E production by the naked DNA

We have investigated immune responses by gene immunization with plasmid DNA encoding major HDM allergen (Der p 1,2,3, Der f 1,2, and 3) to challenges with whole HDM crude extract in mice to mimic realistic clinical setting. We have demonstrated that gene vaccination indeed induced the strong TH1 immune responses, which reduced the IgE antibody production and allergic responses against HDM.

METHODS

Mice

20 BALB/c mice at the age of 6-8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Tennessee (Memphis, TN.) This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animal Welfare Act (7 U.S.C.et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tennessee.

Plasmid construction

Total mRNA was isolated from Der P and Der f HDM, respectively. By using murine leukemia virus reverse transcriptase and random hexanucleotide primer following the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchberg, NJ), first-strand cDNA was generated from 1 μg of total RNA and subjected to RT-PCR. The cDNA was used PCR using Taq polymerase with

primers specific for Der p 1-3, & Der f 1-3. These primers cover the mature excreted region of each genes and include EcoR1 and Xb1 site for cloning and used in this study are summarized in Table 1. The amplified PCR products were subcloned into pcDNA3.1 eukyrotic expression vector (Invitrogen, San Diego, CA) and then sequenced.

DNA preparation and vaccination

Each plasmid construct was prepared using Maxi prep (Quiagen, Chatsworth, CA). Mice were vaccinated by injection with 300 μg of pcDNA3.1 blank vector in 100 μl of PBS (control group) or same amount of the mixed naked DNA encoding the major HDM allergens (vaccination group) three times at weekly intervals into muscle (week 0, 1, and 2). To verify expression, mRNA was prepared from muscle of the injected mice, and used for RT-PCR. We found PCR products of HDM major allergen genes from experimental group mice (data not shown).

Immunization and inhalation of allergen to mice

HDM crude extract (Der p and Der f) were dialyzed, concentrated, and dissolved in PBS buffer. HDM allergen was emulsified with an equal volume of complete Freund's adjuvant (CFA) for immunization. Three weeks after last vaccination, mice were sensitized subcutaneusly at the base of the tail with 100 μg of HDM extract in CFA (week 5). The mice were also given an intraperitoneal dose of 300 ng of purified pertussis toxin at 24 and 72 hours after first immunization. Seven days later, the mice were boosted again with the same amount of antigen in

incomplete Freund's adjuvant (week 6). Mice were inhaled intranasal with 10 μg of HDM crude extract six times at weekly intervals from boost (week 6 to 11).

Expression and purification of recombinant Der p 1 peptide

Recombinant Der p 1 peptide was generated to use as antigen. Der p 1 gene were amplified by RT PCR with primers specific for Der p 1(5'-CCG GAA TTC ATG GAA ACT AAC GCC TGC AGT-3' and 5'-TGC TCT AGA TTA GAG AAT GAC AAC ATA TGG ATA TTC-3') and subcloned into pMAL-c2 (NEB, Beverly, MA), prokaryotic expression vector, using EcoR1 and Xba1 sites. Recombinant Der p 1 was expressed in E.coli. By induction with IPTG at an $O.D._{600}$ of 0.5 in liquid culture for 4 h at 37°C. The purification of fusion proteins were performed with amylose resin (NEB, Beverly, MA). Fractions containing recombinant Der p 1 of >95% purity were dialyzed against 1×PBS buffer and lyophilized until use.

Determination of IgE and HDM specific IgG

The bloods from the 6 mice in two groups were collected six times on week 0(first vaccination), 3, 5(first immunization), 7, 9, and 11. The HDM specific IgG were determined by ELISA as follows. One hundred microliter of HDM (5 μg/ml in 0.1 M carbonate buffer, pH 9.6) were dispensed in each well of a polystyrene microtiter plate (Cost, Cambridge, MA) and incubated overnight at 4°C. The antigen-coated plates were washed three times in 0.05% PBS-Tween 20 buffer (washing buffer) and incubated with mice sera overnight at 4°C. The plates were washed five times with washing buffer and incubated with peroxidase conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO) overnight at 4°C. The plates were washed five times before adding citric acid-phosphate buffer(pH 5.0) containing 0.15 mg/ml of O-phenylenediamine (Sigma, St.Louis, MO). The color was developed at room

temperature, and the reaction was stopped by 2.5M sulfuric acid. The color was measured at 492nm(Bio-Rad, Richmond, CA). The total IgE level was determined by ELISA as follows. One hundred microliter of anti-mouse IgE capture mAb (clone R35-72; Pharmingen, San Diego, CA) were added in each well to plate and incubated overnight at 4°C. After washing, two hundred microliter of 10% fetal calf serum were incubated at room temperature for 30 min. The plates were washed five times with washing buffer and incubated with the diluted mouse serum overnight at 4°C, followed by the addition of one hundred microliter of HRP-conjugated anti-mouse IgE detection mAb (clone R35-118; Pharmingen, San Diego, CA) overnight at 4°C. After washing, color was developed as same as IgG level determination. The purified mouse serum (Pharmigen, San Diego, CA) was used for total IgE standard. In the measure of the HDM specific IgE, the plate were coated with 25 μg/ml HDM in 0.1 M carbonate buffer (pH 9.6) and serum samples were diluted fivefold in 10% FCS. The other procedure were same as the measurement of HDM-specific IgG. The level of HDM-specific IgE were referenced to the standard serum pooled from six mice that were immunized with 100 μg of HDM twice and inhaled with 10 μg of antigen six times. The standard serum was calculated as 100 ELISA units/ml.

Immunohistochemical staining for CD4+ and CD8+ T cells in Lung.

The lung tissues from the experimental and control group mice were removed after the final intranasal inhalation. The tissues were fixed with periodate-lysine-paraformaldehyde solution for 24 h at 4°C. The specimens were rinsed with 0.01 M of PBS (pH 7.4), containing 10% to 20% sucrose, for 36 h at 4°C, embedded in OCT compound (Miles Laboratories Inc., Elkhart, IN), and immediately frozen. The lung

specimen was immersed into 10% EDTA and decalcified for ten days at 4°C. Frozen sections cut at 4 to 6 um in thickness were dehydrated and rinsed in cold PBS. The endogenous pseudoperoxidase was blocked with absolute methanol containing 0.5% hydrogen peroxide for 20 min at room temperature. The sections were treated with 10% normal goat serum in PBS to reduce the nonspecific binding. Biotin conjugated rat anti-mouse CD8 or CD4 monoclonal antibody (Pharmingen, San Diego, CA) diluted to 1:200 in PBS containing 0.5% bovine serum albumin was applied to the sections and incubated overnight at 4°C. After rinsing, the sections were incubated with avidin-biotin peroxidase complexes (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature and rinsed sufficiently with PBS. The reaction was developed with 0.02% 3,3'-diaminobenzidine in 0.05 M of Tris buffer (pH 7.6) with 0.005% hydrogen peroxidase for 7 min. The sections were dehydrated, cleared in xylene, and mounted.

Histological examination of lung tissue

Mice were anesthetized with a mixture of ketalar (35mg/ml), rompun (0.6%/ml) and atropine (0.1mg/ml), of which 0.2 ml was injected intramuscularly. The vascular bed of the lungs was perfused with 0.01M Phosphate-buffered saline (PBS) and then with 4% paraformaldehyde 0.1M PBS buffers. Whole lungs were taken out and were stored in 4% paraformaldehyde for 24 h at 4°C. After fixation, these tissues were dehydrated and embedded in paraffin. Frozen sections cut at 3 um in thickness were stained by hematoxylin and eosin. After coding, the sections were evaluated by two observers using light microscopy. The amount of inflammatory cells per section was scored using the method described by Mehlhop et al. Lungs that

showed no focal inflammation were scored as grade 0. Those that showed one or two centrally located microscopic foci of inflammatory infiltrate were graded as 1. In grade 2, a dense inflammatory infiltrate was seen in a perivascular and peribronchial distribution originating in the center of the lung. In grade 3, the perivascular and peribronchial infiltrates extended to the periphery of the lung.

Measuring cytokine mRNA expression

Mice from two groups were sacrificed 10 days postboot. The lymph nodes were removed from mice and stimulated with recombinant Der p1 (100 μg/ml) or HDM crude extract (100 μg/ml) in vitro for 18 h. the cells were washed with PBS buffer and mRNAs prepared (Biotecx, Houston, TX). By using murine leukemia virus reverse transcriptase and random hexanucleotide primer following the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchber, NJ), first-strand cDNA was generated from 1 μg of total RNA and subjected to RT-PCR analysis. To determine the relative abundance of each cytokine mRNA expression, the amount of each cDNA for PCR were optimized by the intensity of the amplified DNA products of β-actin from each RNA. In the PCR reaction mixture, either β-actin as control primer, IL-2, IFN-γ (Clonetech, PaloAlto, CA), IL-4, IL-5, IL-10 at the final concentration of 0.2 μM were added. The PCR condition was as follows: 200 μM of dNTP, 10 μCi [32P] dCTP, 50 μM Tris. HCl (pH 9.0), 50 μM NaCl, 2 μM MgCl2, 0.5 mM DTT, and two units of Taq polymerase (Perkin Elmer, Branchberg, NJ) at a final volume of 20 μl. A negative control reaction was run with each sample to verify that no PCR bands appeared in the absence of template. The optimal amplification conditions were as follows: 45s at 94°C for denaturation, 45s at 67°C for annealing,

and 1 min at 72°C for elongation and the PCR cycles were 30. The amplified DNAs of β-actin, IFN-γ, IL-2, IL-4, IL-5, and IL-10 had sizes of 540, 365, 413, 354, 349, and 455 base-pair, respectively. The gel was dried on whatman 3M paper and exposed to Kodak XAR film. In each electrophoresis run, intra-and inter-gel staining homogeneity was confirmed by staining intensity of molecular weight markers at both ends of the gels. In general, amplification kinetics was monitored for each PCR run by examining aliquots of the products on the gel. Amounts of the PCR products were compared during the cycle where the amplification did not reach saturation.

Statistical analysis

Data in immunoglobuline response were analyzed by Student's paired *t* test for comparisons between control and experimental group. Histological grades were analyzed using a non-parametric Wilcoxon test. Data were expressed as mean±SD. A *P* value< 0.05 was considered significant.

RESULTS

Downregulation of Der p-specific IgE antibody production by gene vaccination.

To examine the immune response, we checked the IgG and IgE antibody productions by ELISA (Fig 1). The gene vaccination with the major HDM allergen genes, Der p1, 2, 3, Der f1, 2, & 3, showed about 70% inhibition of HDM-specific IgE and more than 70% inhibition of total IgE as compared with the control group after 6 weeks immunization. Thus, genetic vaccination could inhibit an in vivo

allergen-specific IgE synthesis efficiently even though HDM-specific IgG antibody productions of both group showed an almost same level (Fig 1A-C).

Histological and immunohistochemical study

To examine whether the genetic vaccination effect on the cellular response of lung or not, we stained the lung at the end of the experiment by histological and immunohistochemical method. The lung from the control group (mean grade 1.64 ± 0.52) showed the much more infiltration of inflammatory cells in the submucosa of airways than that of the vaccination group (mean grade 0.68 ± 0.48) (Fig 2A,B, and C). Also eosinophils were detected in the lung of the control mice (Fig 2D). In the immunohistochemical stain for CD4+ & CD8+ T cells showed that the more CD8+ T-cells infiltrated in the submucosa and mucosa of airway of the lung from the vaccination group as compared with the control group (Fig 3). But in the stain for the CD4+ T cells showed no difference the two groups. So the results suggested that the genetic vaccination also effect on the cellular response and the CD8+T cells of the vaccination were capable of protection against a subsequent allergenic challenge.

Cytokine gene expression by antigen stimulation in vivo

To determine whether the Th1 or Th2 cytokine involve in the genetic vaccination, T-cells were harvested from lymph node of the two group mice and stimulated with recombinant Der p1 or HDM crude extract in vivo. A higher mRNA expression of IFN-γ in the vaccination group was detected compared with the control group. But in the mRNA expression of Il-2, 4, 5, & 10 they did not showed different

responses from the control group. These data indicate that the vaccinations with HDM major genes induced a strong Th1 cytokine (IFN-γ) gene expression in lymph node.

DISCUSSION

Diseases such as allergic asthma and rhinitis, and atopic dermatitis are all characterized by elevated levels of serum IgE. Total and Specific IgE positivity also showed a close relationship with clinical symptom in atopic allergy [15]. A variety of approaches targeting the suppression of IgE have been proposed as synthetic peptides as T-cell vaccine. However, synthetic peptides were poor immunogen and were needed higher than the amount derived intracelluarly from processed antigens. Recently Hsu et al.[10)] showed that gene immunization of rats with plasmid encoding Der p 5 prevent induction of IgE synthesis. These data suggest that pDNA immunization with containing the gene for the minor HDM allergen Der p 5 may induce Th1 immune responses to the encoded antigens. The Der p 5 allergen react with about only 40% allergic sera but the Der p 1 and 2 allergens react with about 80% of allergic sera. We have analyzed the effects of gene vaccination with plasmid encoding major 6 HDM allergens (Der p 1, 2, & 3, Der f 1, 2, & 3) in allergic responses to whole HDM crude extract. Our results showed about 50% inhibition of HDM-specific IgE and more than 70% inhibition of total IgE at week (end of the study)(Fig 1). Thus, this result suggested that gene immunization with plasmid encoding the major HDM antigen can also induced inhibition of IgE synthesis. Animal models have established that Th2 responses are mediated by T helper cells that secret cytokines such as IL-4, IL-5 and IL-10 that induce antibody production in B cells, above all, formation of IgE as playing a central role in allergic responses.

IFN-γ is the Th1 cytokine responsible for the inhibition of IL-4-mediated IgE responses and promotes the formation of IgG2a. Previous report showed that protein immunization induced a TH2 response, as shown by IgG1 and IgE antibody formation and IL-4 & IL-5-secreting T cells. In contrast, gene immunization with plasmid DNA induced a TH1 response with IgG2a antibody production and IFN-γ secreting T cells. Genetic vaccination in many infectious disease, and allergic disease enhanced TH1 response for preventing several diseases. Our gene vaccination data showed that the mRNA expression of IFN-γ in lymph node from pDNA encoding the HDM allergen Der p 1,2,3, & Der f 1,2,3 gene increased more than that from the control group. This data suggested that the genetic immunization might induce TH1 immune response to the encoded antigen or allergen. After genetic immunization, the TH1 response dominated over the TH2 response and downregulates preexisting IgE antibody formation. Our experiment suggested that genetic immunization might suppress IgE production by the inducing the TH1 response from T helper cells.

Allergic asthma is characterized as a chronic inflammatory disease of the bronchi and it is well established that a variety of cells including mast cells, eosionphils and lymphocytes play a role in this process. After an inhalation challenge, the inflammatory cells migrate from the peripheral blood to the site of inflammation in the bronchial mucosa and bronchoalveolar fluid showed dominant TH2-type cytokines. Our histological study showed that gene vaccination induced the reduction of infiltration of inflammatory cells in lung tissues (Fig 2). It suggested that the change in the function of T cells might effect on the reduction of the inflammatory cells in bronchial mucosa. This data indicated that gene immunization affects not only humural immune rcsponses but also cellular responses. T lymphocytes have been

suggested to play a key role in orchestrating the interaction of the participating cells since they are able to release an array of cytokines which can attract, prime and activate other cell types. A successful outcome of immunotherapy has been associated with the development of suppressor T cells, which can downregulate the allergic response. Recent data have also revealed that functionally distinct subsets of CD8+ T cells may play an important regulatory role in IgE production. But Manickan et al. showed that the mechanism of genetic immunization was principally by CD4+ T cells, but not by CD8+ T cells. Recently Lee et all reported that both CD4+ and CD8+ subsets of T cells from pDNA immunized mice can suppress IgE antibody production by affecting the primary response and or by propagating the Th1 memory response in a passive cell transfer system. Our immunohistochemical study showed more CD8+ T cells were detected in the lung of the vaccinated group than that of control group (Fig 3). Peptides derived from extracellular molecules are presented to CD4+ T cells by MHC class II molecules normally generated by antigen-presenting cells [36], whereas peptides derived from cytosolic proteins are generally presented to CD8+ T cells by major histocompatibility complex (MHC) class I molecules which are expressed on virtually all somatic cells. We injected with the mixed naked DNA into muscle of BALB/c mice. It suggested that such endogenous production of an allergenic protein might be a useful means to induce regulatory CD8+ T cells capable of conferring protection against a subsequent allergenic challenge. Our represented results here showed that vaccination with plasmid DNA encoding specific allergen genes in animal model provided an efficient clinical method for modulation allergic responses.

Table 1

Oligonucleotides used for Der p1-3 & Der f 1-3 in this study

Molecule Primer	Sequence (5' to 3')
Der p 1	5'-CCG GAA TTC GCC GCC ACC ATG GAA ACT AAC GCC TGC AGT ATC AAT GGA TGC TCT AGA TTA GAG AAT GAC AAC ATA TGG ATA TTC-3' [SEQ.ID.NO:11]
Der p 2	5'-CCG GAA TTC GCC GCC ACC ATG GAT CAA GTC GAT GTC AAA GAT TGT GCC TGC TCT AGA TTA ATC GCG GAT TTT AGC ATG AGT AGC AAT-3' [SEQ.ID.NO:12]
Der p 3	5'-CCG GAA TTC GCC GCC ACC ATG ATT GTT GGT GGT GAA AAA GCA TTA GCTG TGC TCT AGA TTA CTG TGA ACG TTT TGA TTC AAT CCA ATC GATA-3' [SEQ.ID.NO:13]
Der f 1	5'-CCG GAA TTC GCC GCC ACC ATG GAA ACA AGC GCT TGC CGT ATC AAT TCG TGC TCT AGA TTA GAG GTT GTT TCC GGC TTG GAA ATA TCC G-3'[SEQ.ID.NO:14]
Der f 2	5'-CCG GAA TTC GCC GCC ACC ATG GAT CAAA GTC GAT GTT AAA GAT TGT GCC TGC TCT AGA TTA ATC ACG GAT TTT ACC ATG GGT AGC AAT-3' [SEQ.ID.NO:15]
Der f 3	5'-CCG GAA TTC GCC GCC ACC ATG ATT GTT GGT GGT GTG AAA GCA CAA GCC TGC TCT AGA TTA CTG TGA ACG TTT TGA TTC AAT CCA ATC GAC-3' [SEQ.ID.NO:16]

Table 2

Oligonucleotides used for cytokine mRNA expression in this study

Molecules	Primer Sequence (5' to 3')	Product Size
β-actin	5'-GTG GGC CGC TCT AGG CAC CAA CTC TTT GAT GTC ACG CAC GAT TTC-3' [SEQ.ID.NO:17]	540bp
IL-2	5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG GACAGAAGGCTATCCATCTCCTCAGAAAGTCC-3' [SEQ.ID.NO:18]	413bp
IFN-γ	5'-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC TGGACCTGTGGGTTGTTGACCTCAAACTTGGC-3' [SEQ.ID.NO:19]	365bp
IL-4	5'-CAG CTA GTT GTC ATC CTG CTC TTC GTG ATG TGG ACT TGG ACT CAT TCA TGG-3' [SEQ.ID.NO:20]	357bp
IL-5	5'-TGT CTG GGC CAC TGC CAT GGA GAT TC CCA TTG CCC ACT CTG TAC TCA TCA CAC-3' [SEQ.ID.NO:21]	424bp
IL-10	5'-ATG CAG GAC TTT AAG GGT TAC TTG GGT ATT TCG GAG AGA GGT ACA AAC GAG G-3' [SEQ.ID.NO:22]	455bp

Figure Legend

FIG 1. Effect of vaccination on the allergen induced immumoglobulin prodution. The total IgE antibody response (A), and the HDM-specific IgE antibody response (B), and changes of HDM-specific IgG antibody respons (C), of BALB/c mice after immunization of whole HDM crude extract. Data shown are means ± SD (n=6 per group). **P* < .05 compared with the control group.

FIG 2. Histopathologic examination of lung.

Lungs from control and experimental groups of mice were removed on day 45 after immunization. (A). lung from control mouse (×100). (B). lung from control mouse (×200). (C). lung from vaccination mouse (×200). (D). Bronchial wall from control mouse (×600). Eosinophils and many inflammatory cells were observed in

control group. Vaccination mice showed much less the infiltration of inflammatory cells than control mice.

FIG 3. Immunohistochemical examination of lung.

Lungs from control and vaccination group were removed on day 45 after immunization and were stained for CD8+ T cells. (A): lung from control mouse (×100). (B). lung from vaccination mouse (×100). (C). lung from control mouse (×200). (D). lung from vaccination mouse (×200). (E). Bronchial wall from vaccination mouse (×400). The more CD8+ T cells were observed in vaccination mouse.

FIG 4. Cytokine gene expression

T cells were collected from the lymph nodes of control or vaccination mice 10 days post boost and cultured in the presence of no antigen(-), recombinant Der p1(100 μg/ml), and HDM crude extract (100 μg/ml) for 18 h. The total RNA was extracted using TRIzol reagent and RT-PCR reactions were doing using cDNA with different primers specific for β-actin, IL-2, 4, 5, 10 and γ-interferon.

Although only a few exemplary embodiments of this invention have been described in detail above, those skilled in the art will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the following claims.

CLAIMS

CLAIMED IS:

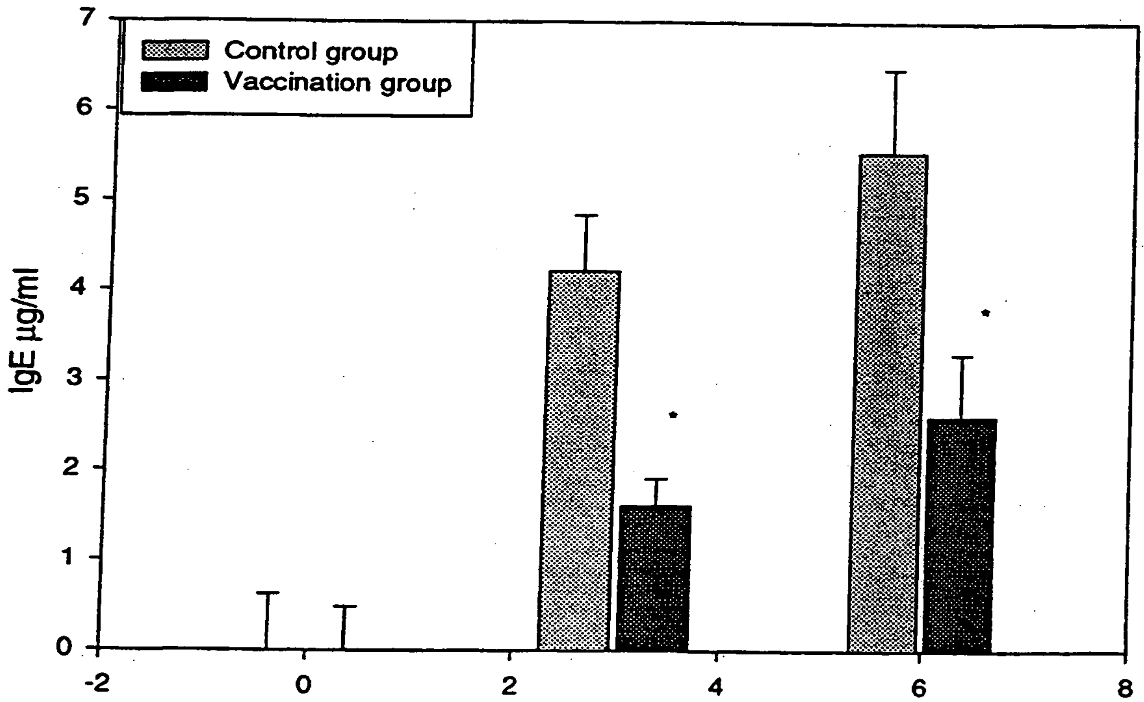
1. A composition for reducing IgE production, comprising: a pharmacologically acceptable medium and a substantially pure, immunogenic plasmid DNA encoding a T cell eptitope.

2. A method of reducing reducing IgE production, comprising administering a composition comprising a pharmacologically acceptable medium and a substantially pure, immunogenic plasmid DNA encoding a T cell eptitope.

3. A vaccine for reducing the severity of an allergic disease in a mammal, comprising a pharmaceutically acceptable carrier and at least one plasmid DNA selected from a group consisting of the house dust mite Dermatophagoides pteronyssimus (Der p) and Dermatophagoides farinae (Der f).

4. A composition for reducing IgE production, comprising: a pharmacologically acceptable medium and a substantially pure, immunogenic plasmid DNA encoding a major HDM allergen selected from a group consisting of Der p 1, Der p 2, Der p 3, Der f 1, Der f 2, and Der f 3.

Changes of total Immunoglobulin E antibody serum levels in BALB/c mice after immunization of Der p extract



Weeks after immunization of Der p extract
*P<0.05 vs control group

FIG. 1A

Changes of Der p specific anti-IgE antibody serum levels in BALB/c mice after immunization of Der p extract

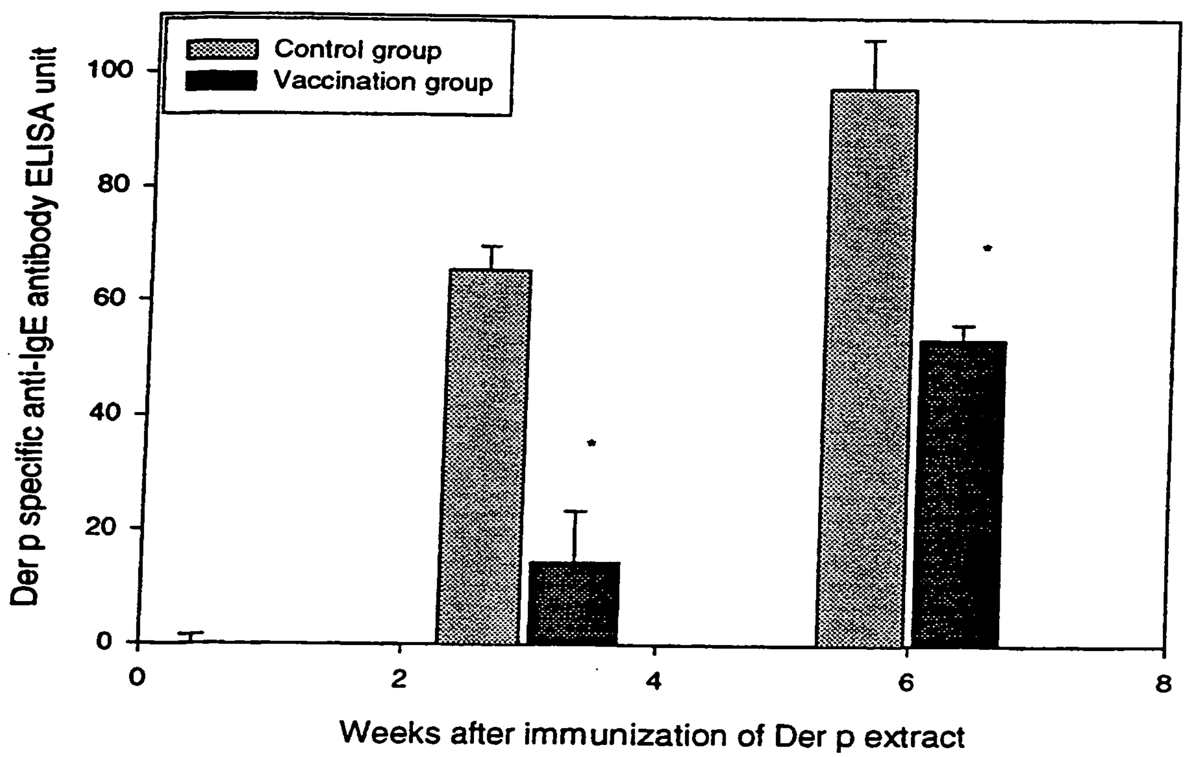
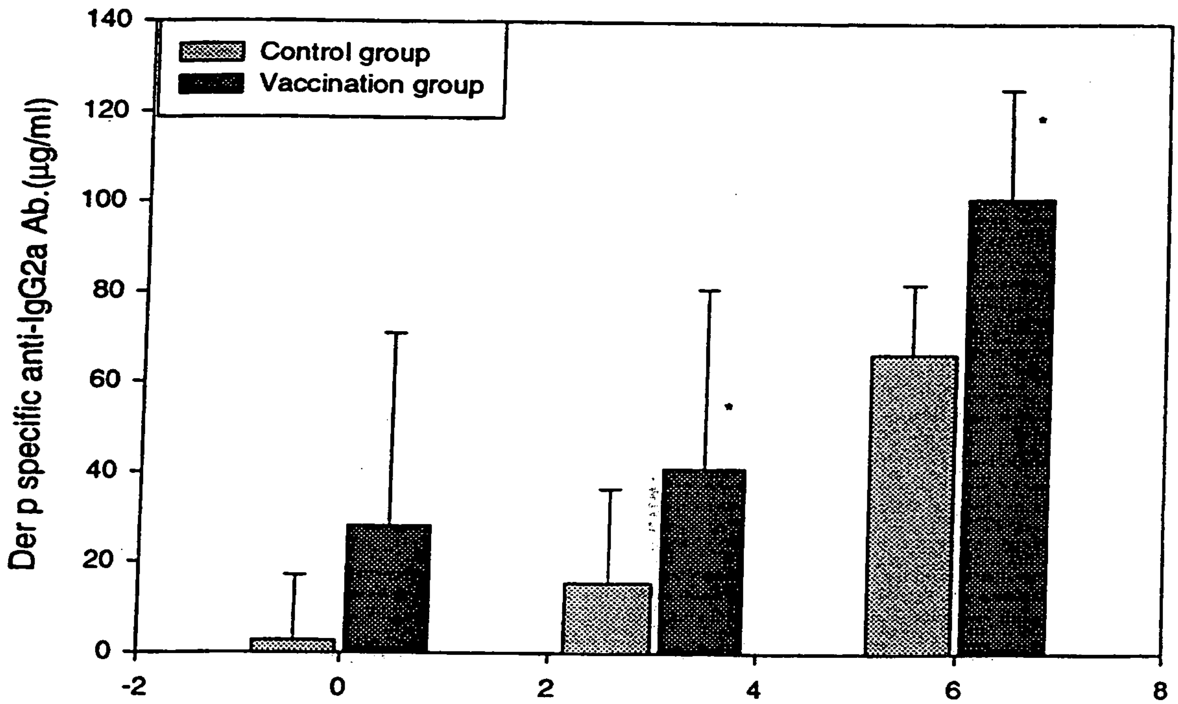


FIG. 1B

Changes of Der p specific anti-IgG2a antibody serum levels in BALB/c mice after immunization of Der p extract



Weeks after immunization of Der p extract
*P<0.05 vs control group

FIG. 1C

Changes of Der p specific anti-IgG antibody serum levels in BALB/c mice after immunization of Der p extract

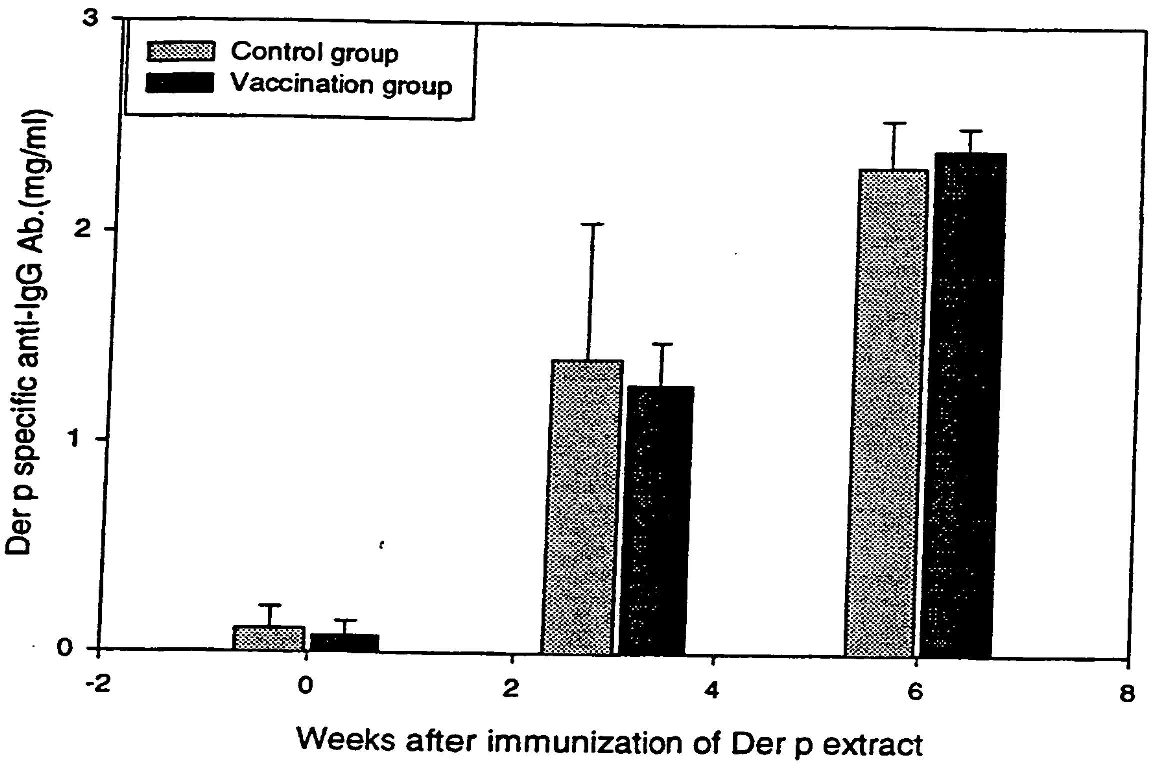
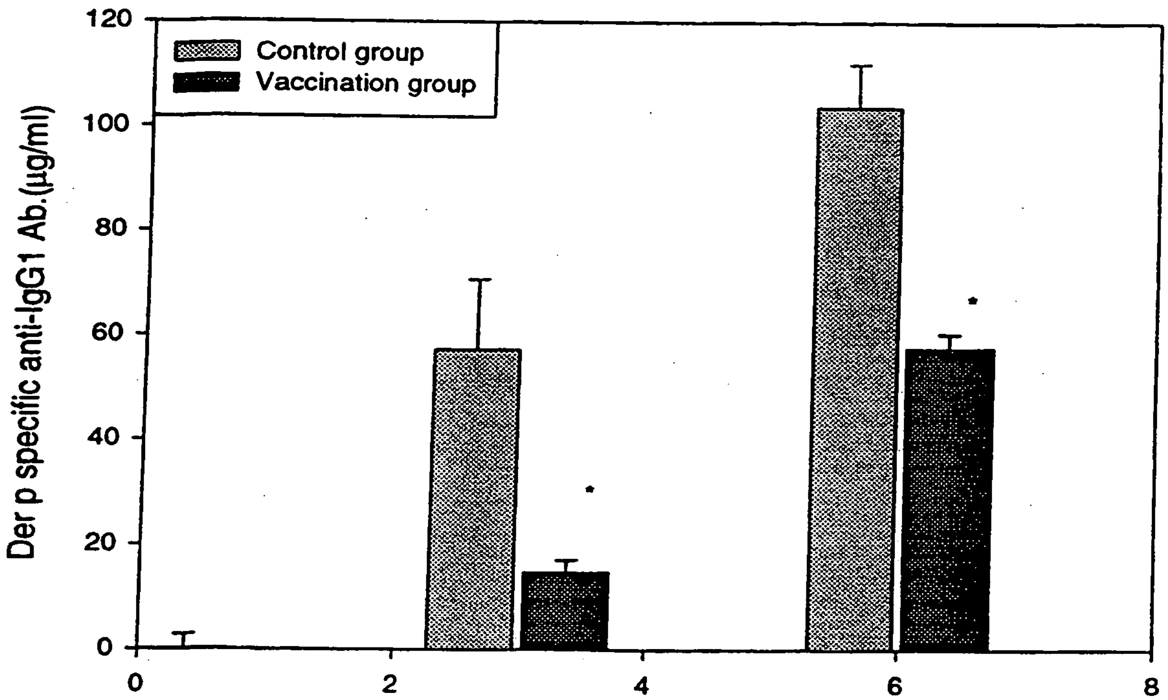


FIG. 1D

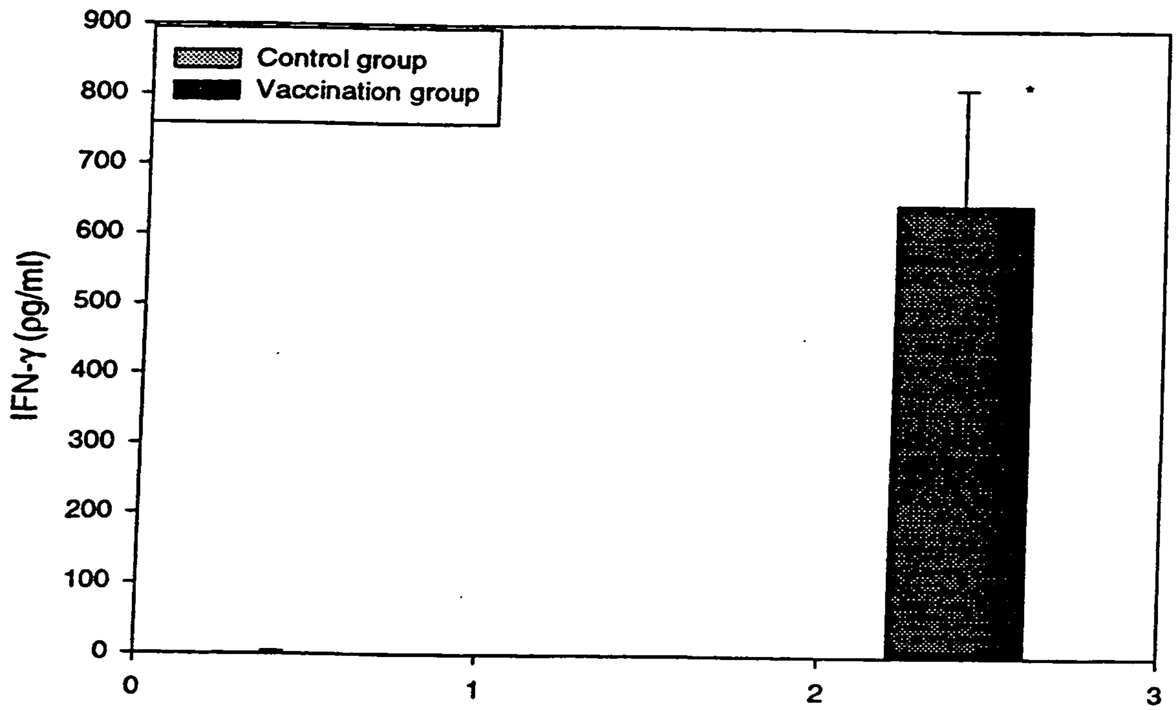
Changes of Der p specific anti-IgG1 antibody serum levels in BALB/c mice after immunization of Der p extract



Weeks after immunization of Der p extract
*P<0.05 vs control group

FIG. 1E

The IFN-γ serum levels in BALB/c mice after immunization of Der p extract



Weeks after immunization of Der p extract
*P<0.05 vs control group

FIG. 1F

The IL-4 serum levels in BALB/c mice after immunization of Der p extract

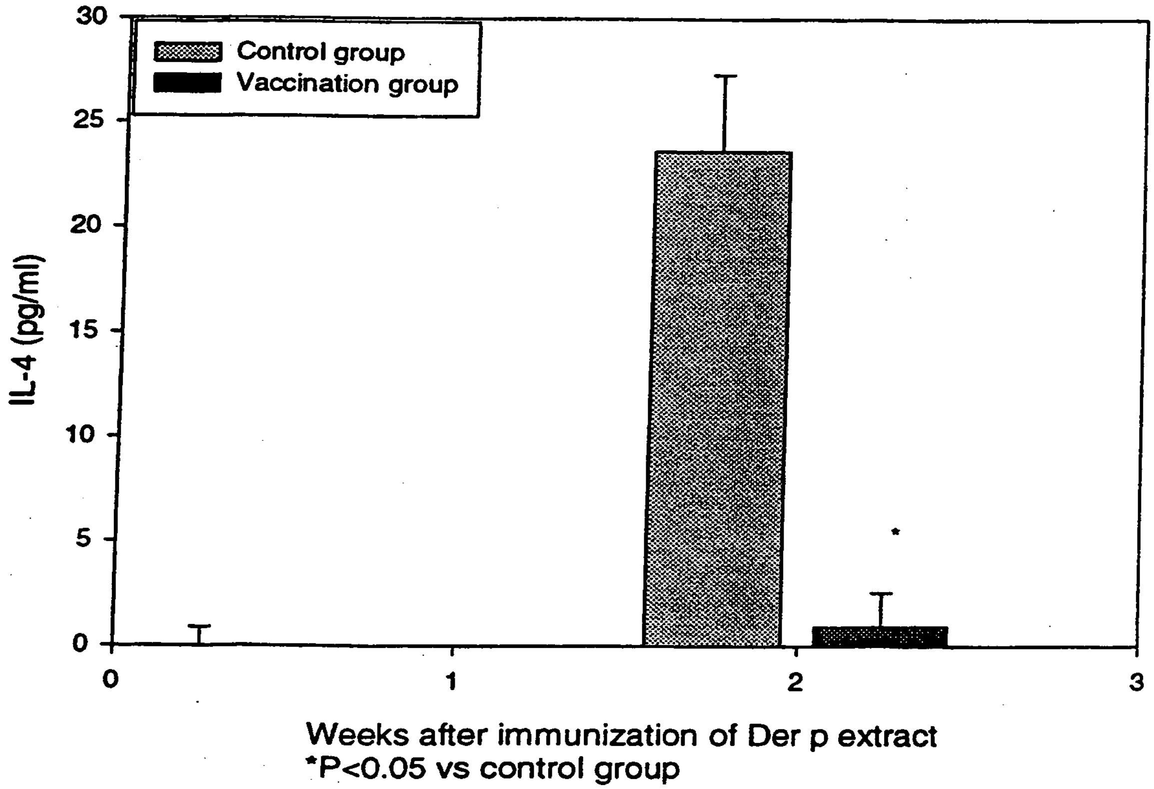


FIG. 1G

Proliferative response in draining lymph node cells from BALB/c mice

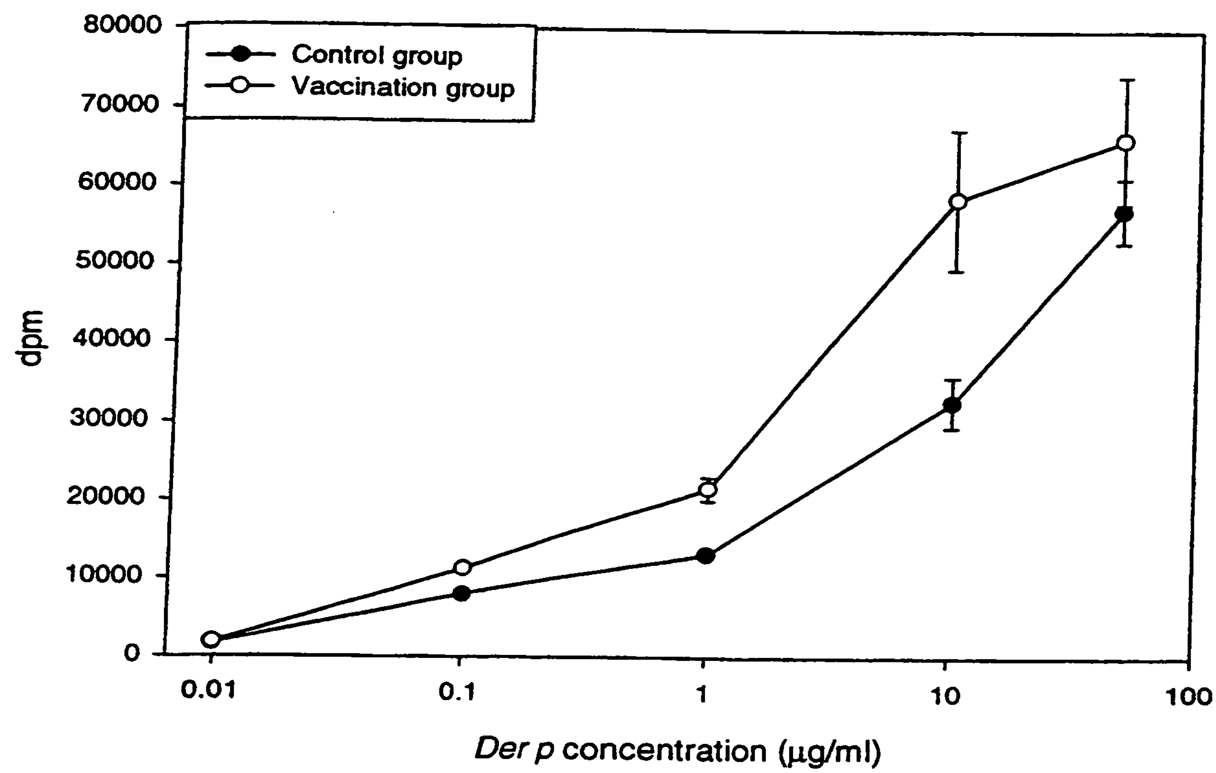
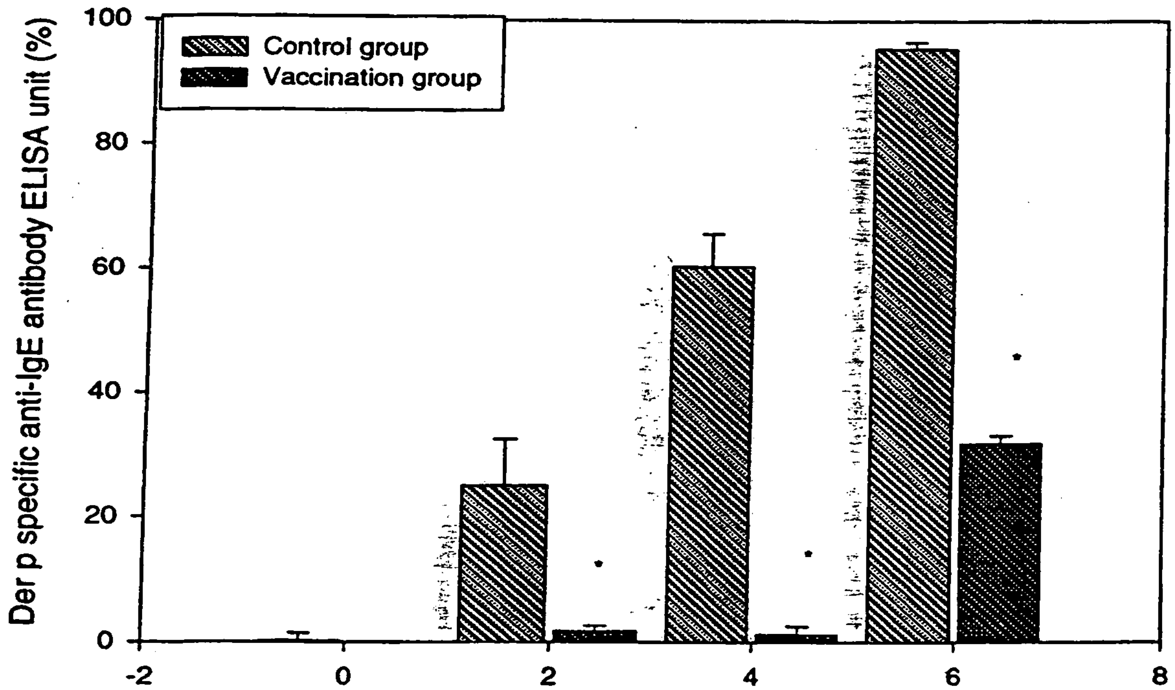


FIG. 1H

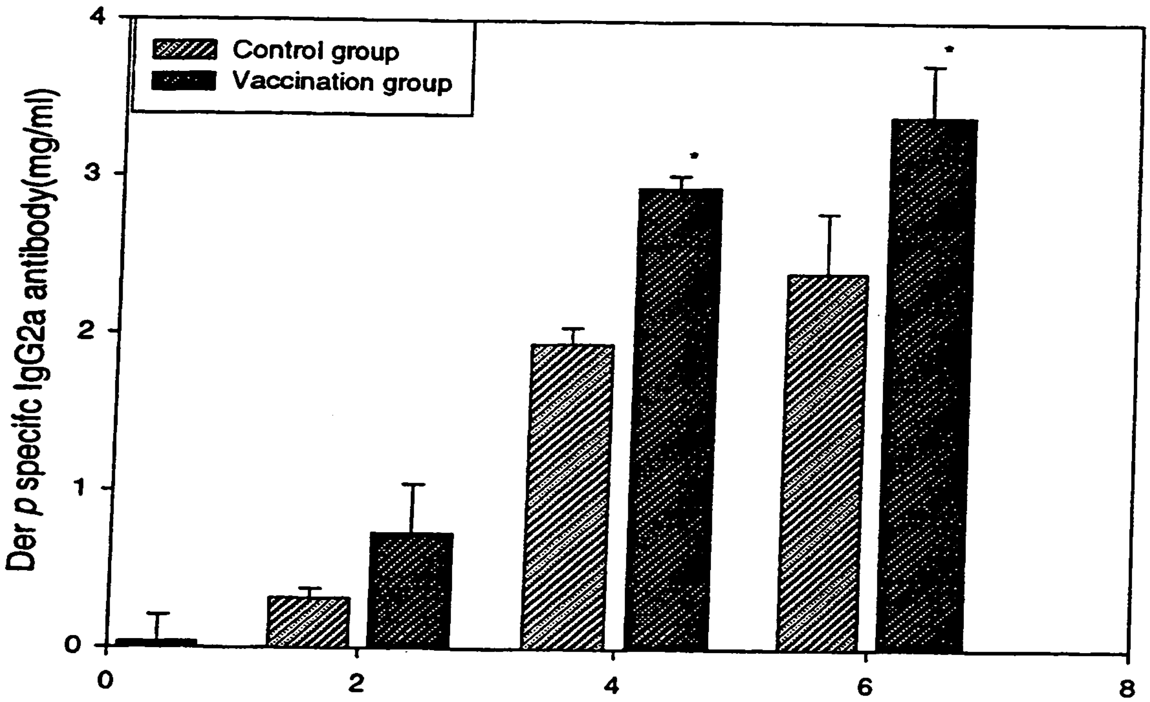
Changes of Der p specific IgE antibody serum levels in BALB/c mice after immunization of Der p extract



Weeks after immunization of Der p extract
* P<0.05 vs control group

FIG. 2A

Changes of Der *p* specific IgG2a antibody serum levels in BALB/c mice after immunization of Der *p* extract



Weeks after immunization of Der *p* extract
* P<0.05 vs control group

FIG. 2B

Changes of Der *p* specific IgG1 antibody serum levels in BALB/c mice after immunization of Der p extract

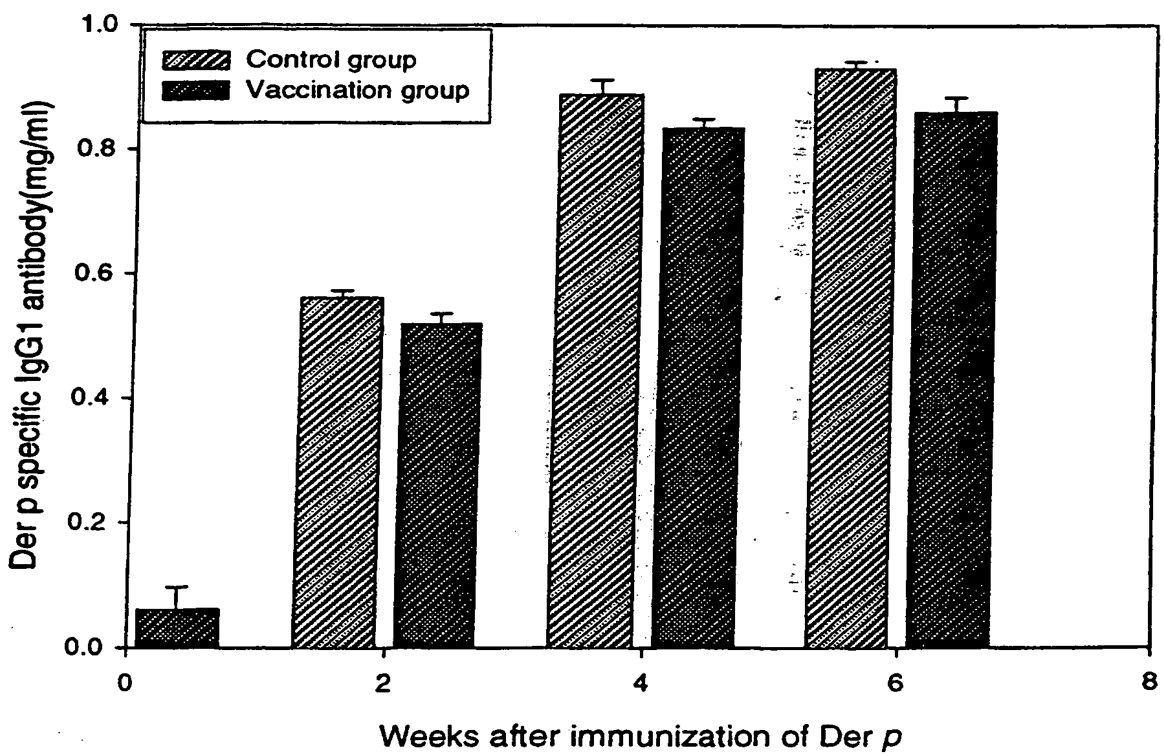


FIG. 2C

Changes of Der p specific anti-IgG antibody serum levels in BALB/c mice after immunization of Der p extract

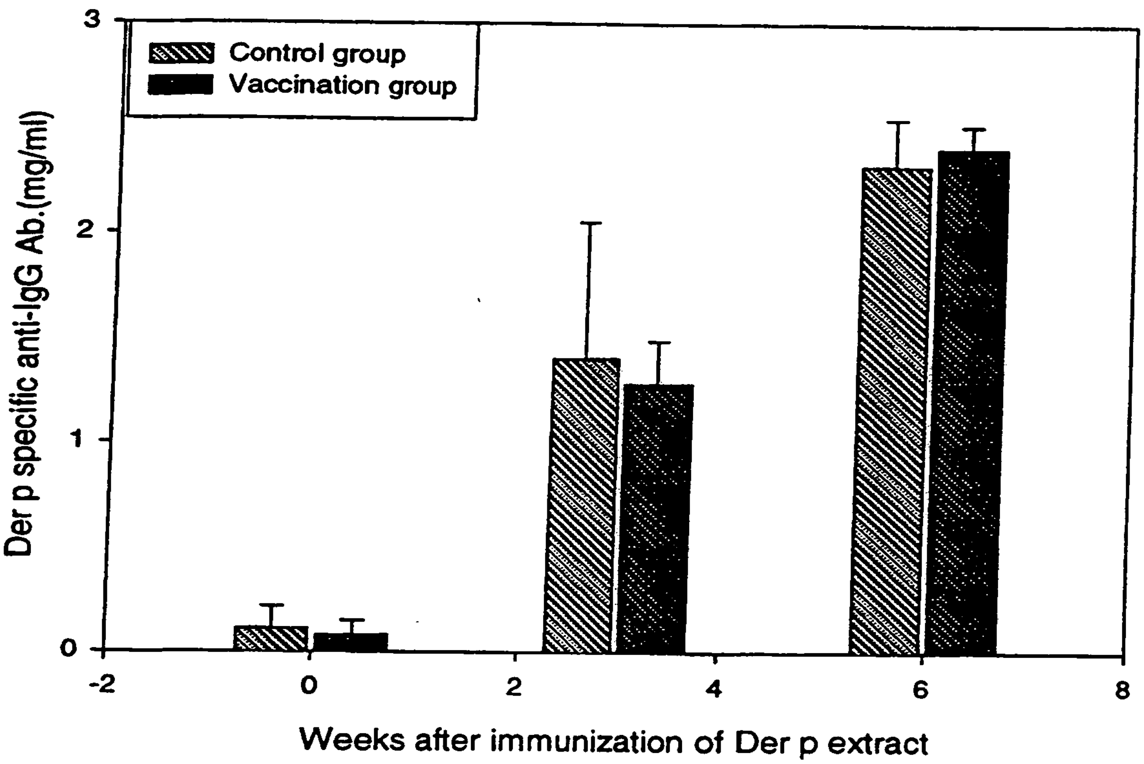


FIG. 2D

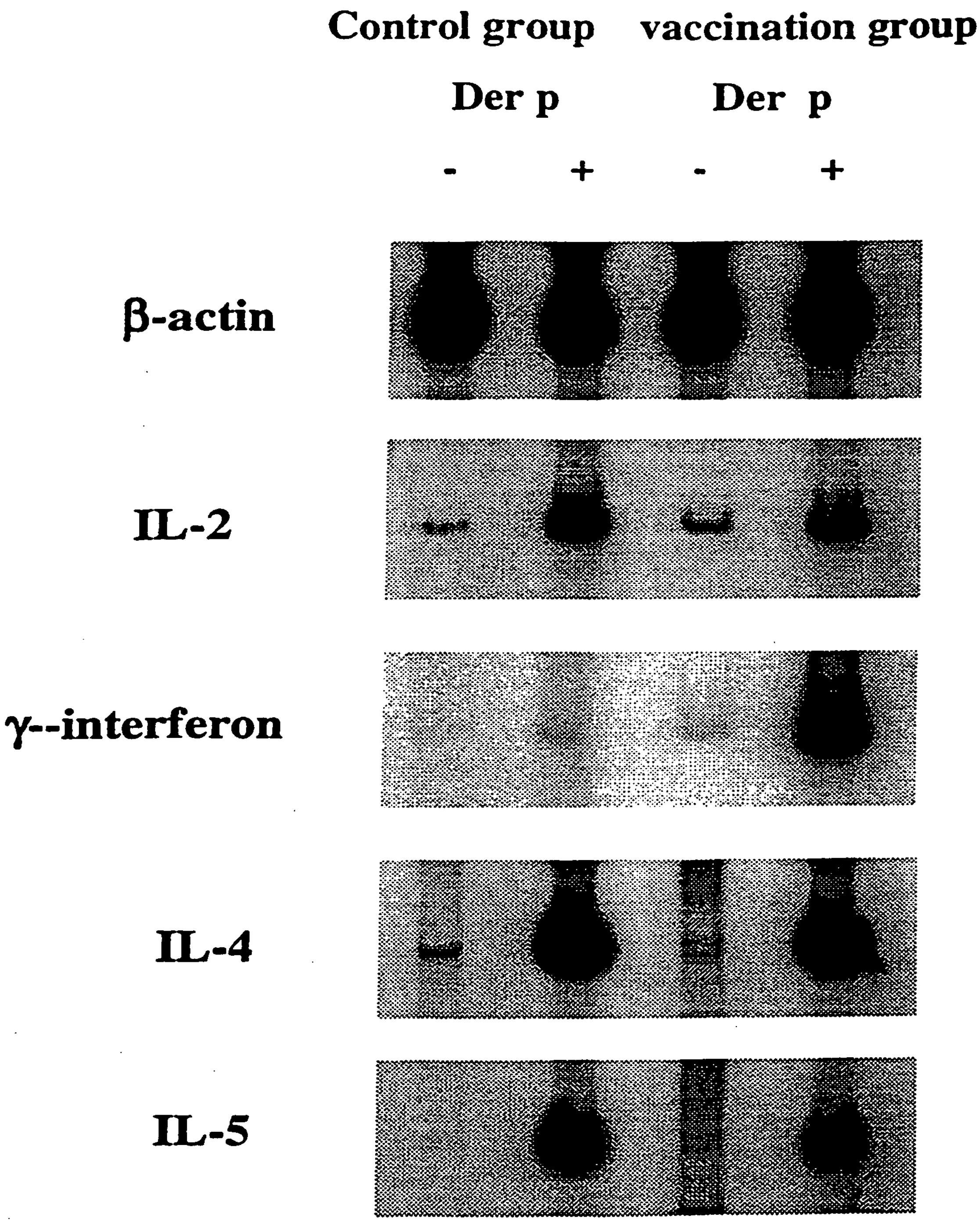

Control group vaccination group
Der p Der p
- + - +
β-actin
IL-2
γ--interferon
IL-4
IL-5

FIG. 2E

Changes of total IgE serum levels in BALB/c mice after i.m. injection of pCDNA

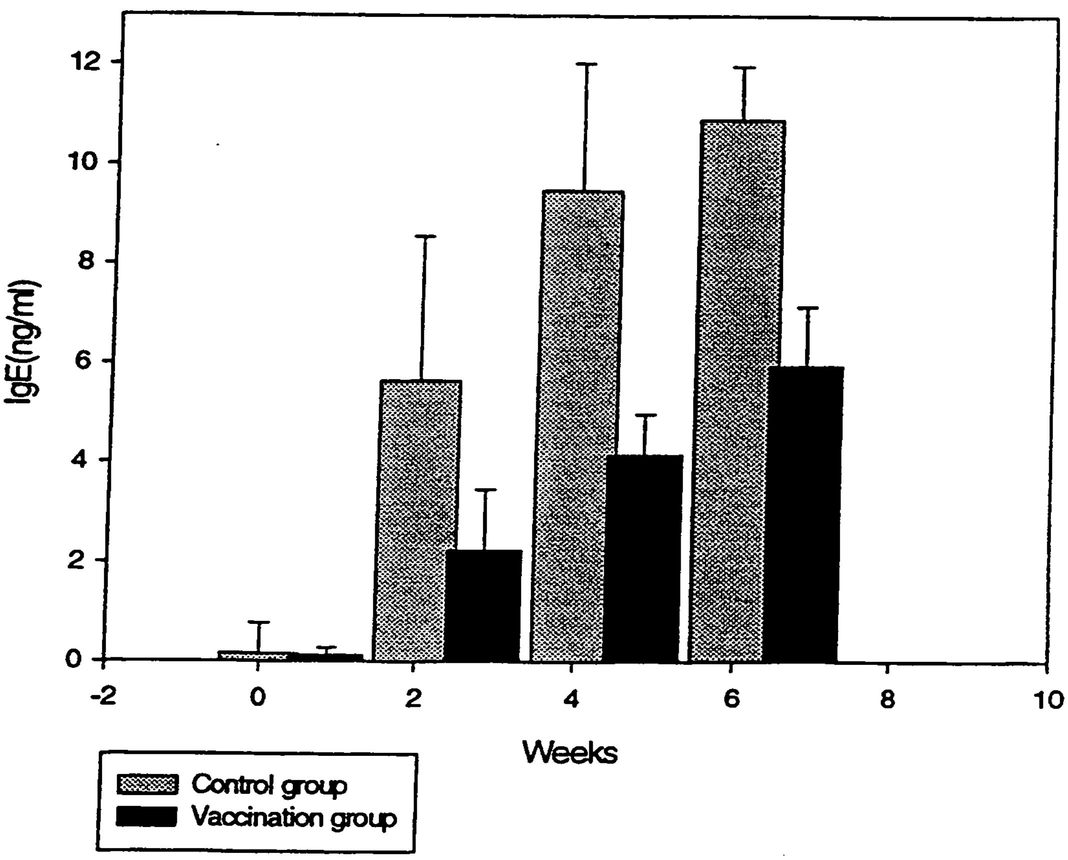


FIG. 2F

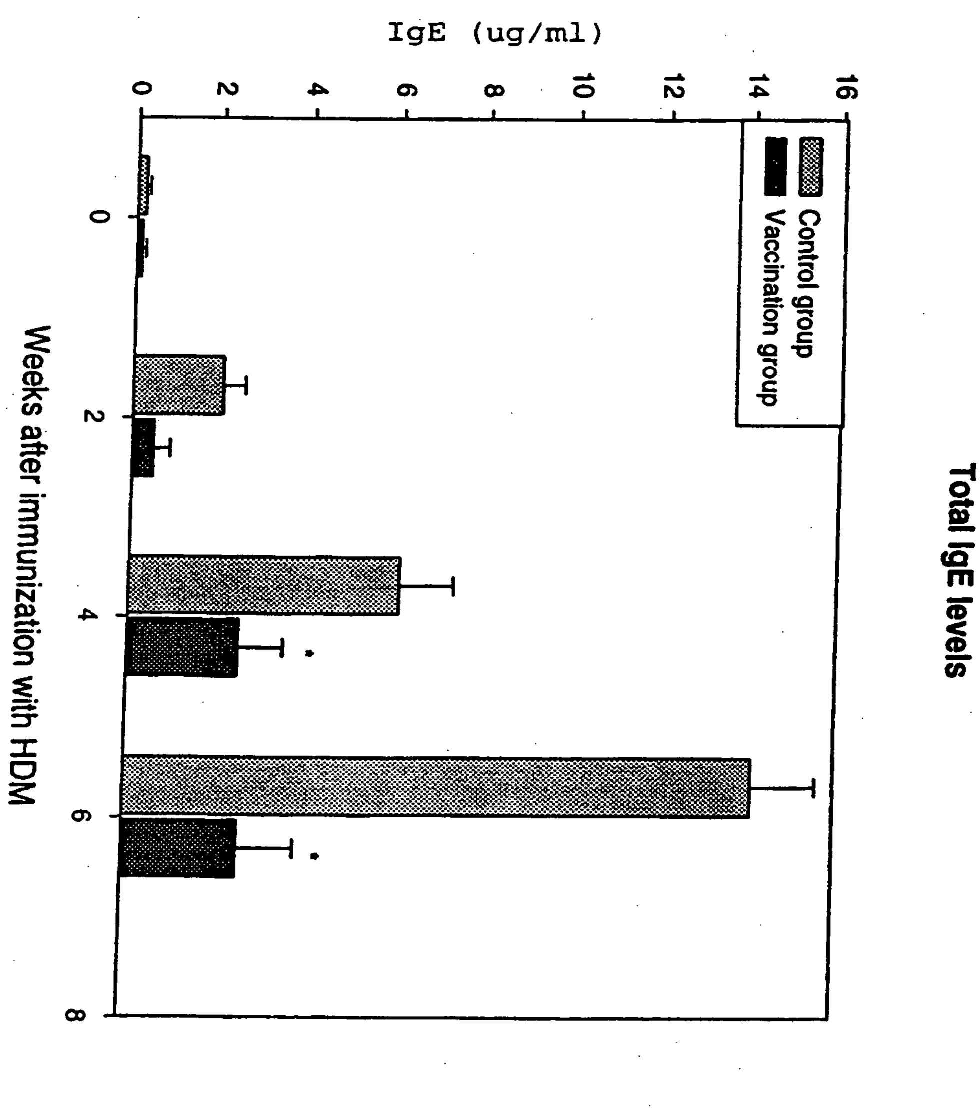

Total IgE levels
Control group
Vaccination group
IgE (ug/ml)
0
2
4
6
8
10
12
14
16
0
2
4
6
8
Weeks after immunization with HDM

FIG. 3A

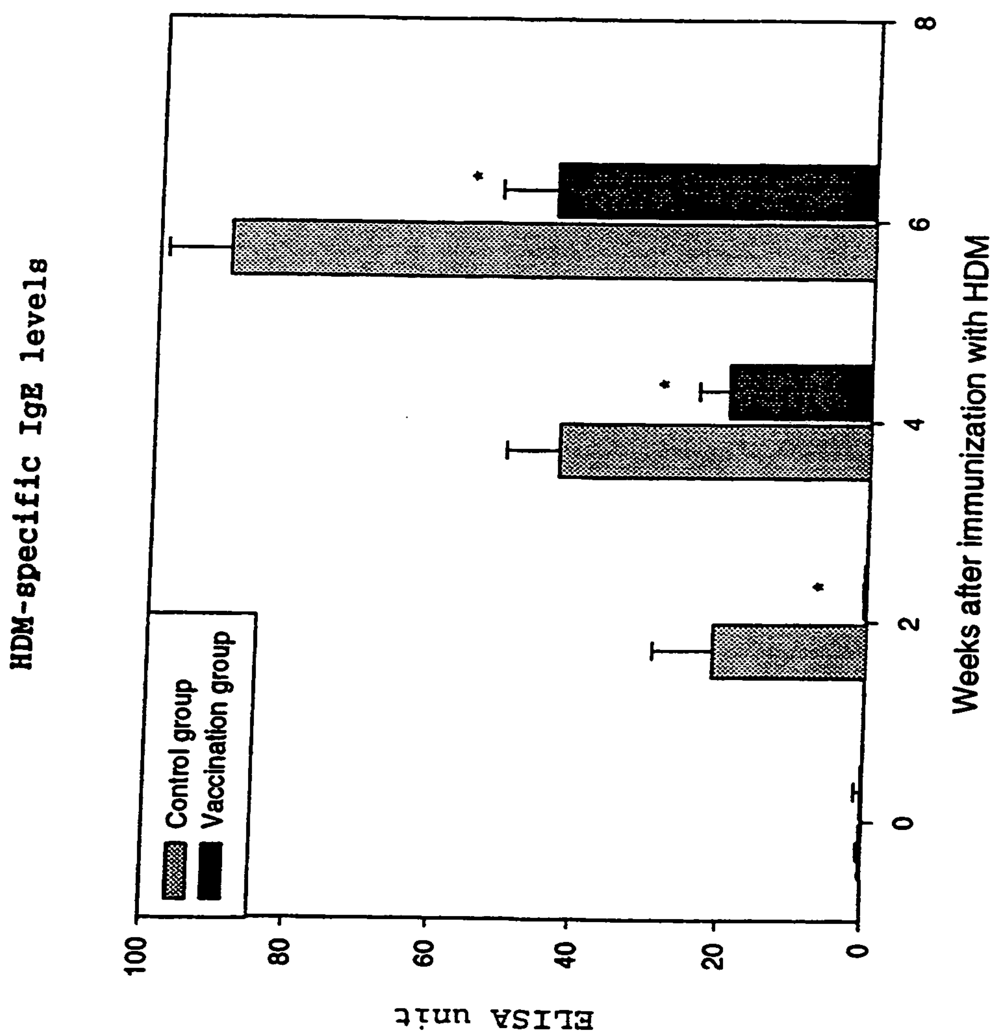

HDM-specific IgE levels
Control group
Vaccination group
ELISA unit
100
80
60
40
20
0
0
2
4
6
8
*
*
*
Weeks after immunization with HDM

FiG3B

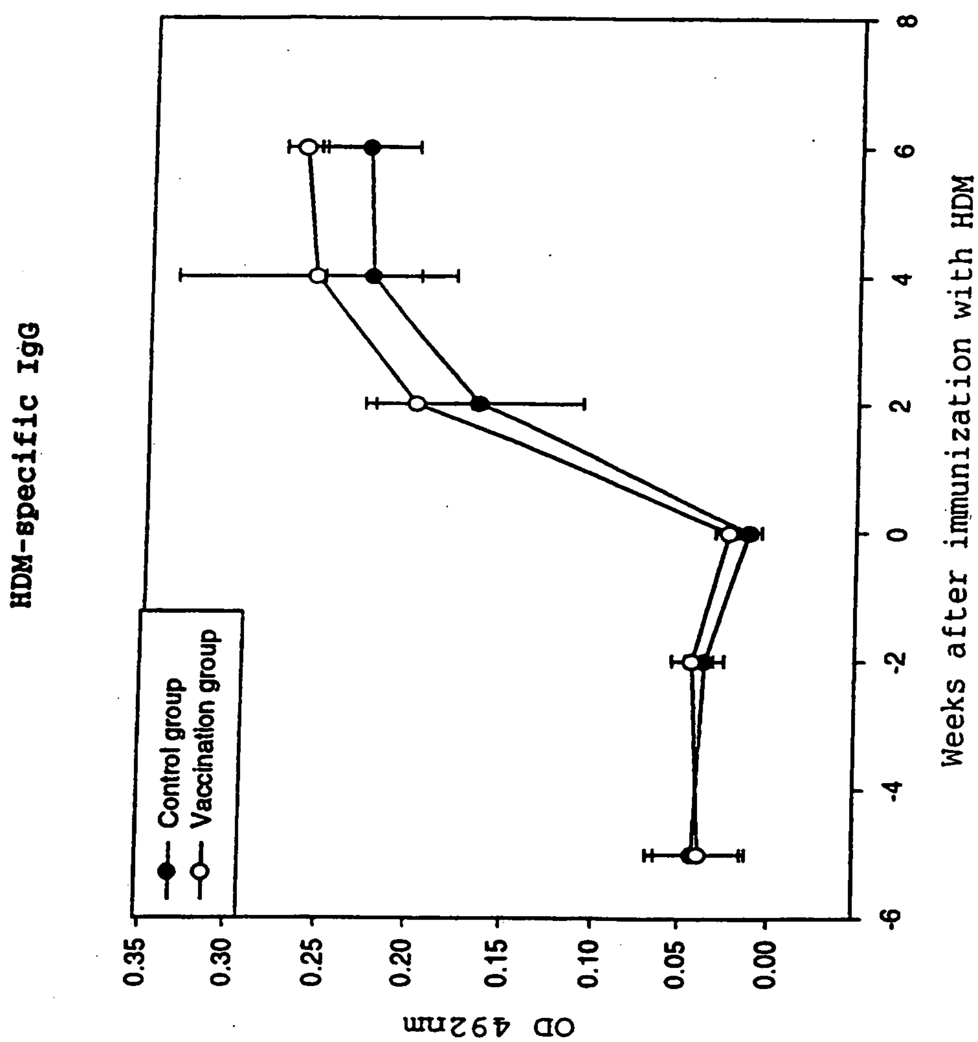

HDM-specific IgG
Control group
Vaccination group
OD 492nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-6
-4
-2
0
2
4
6
8
Weeks after immunization with HDM

FiG 3C

Figure 4

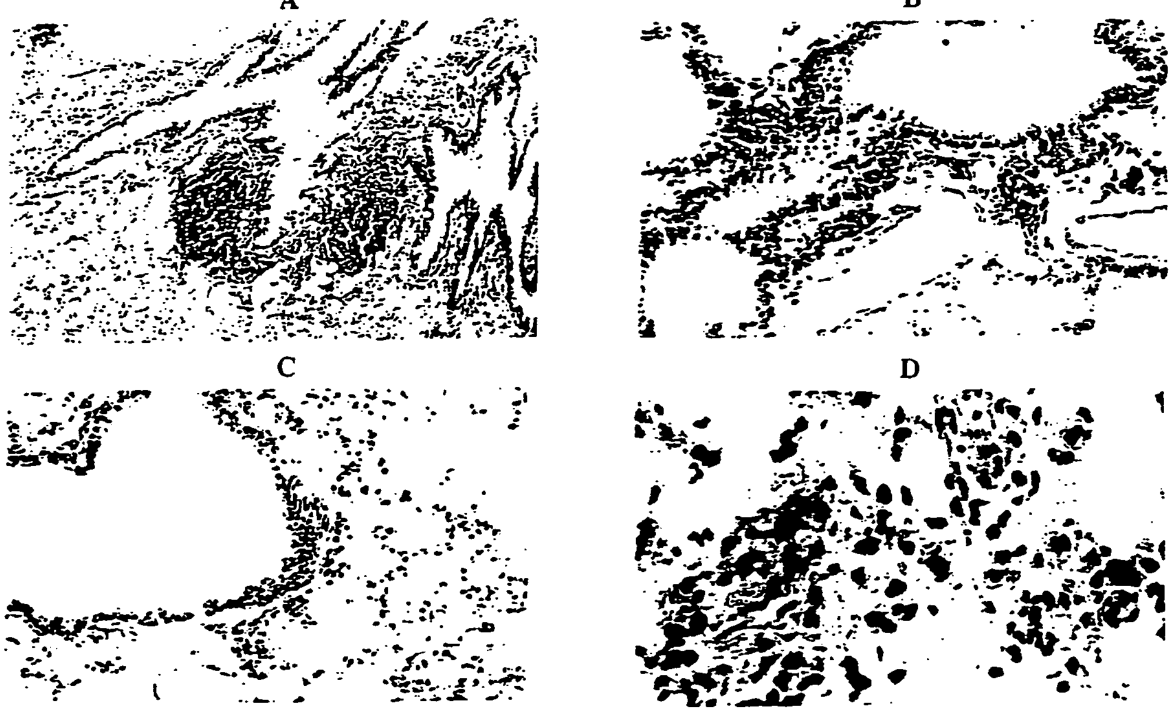
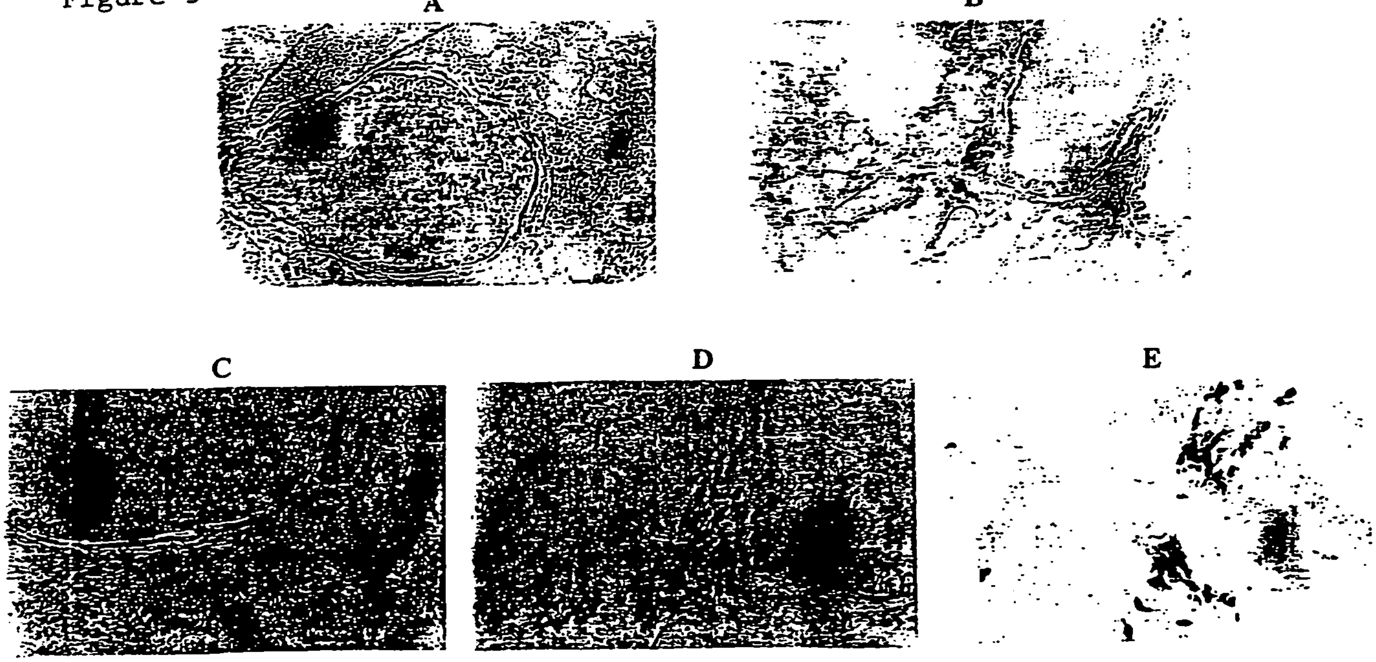


Figure 5



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INTERNATIONAL SEARCH REPORT

Inte[...]onal Application No
PCT/US 00/04780

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/70 A61P11/06 //A61K39/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KWON, S. S. ET AL: "Immunoprotective effect of vaccination with DNA encoding T cell epitopes on the Der p induced IgE production." JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, (JAN., 1999) VOL. 103, NO. 1 PART 2, PP. S110. MEETING INFO.: 55TH ANNUAL MEETING OF THE AMERICAN ACADEMY OF ALLERGY, ASTHMA AND IMMUNOLOGY ORLANDO, FLORIDA, USA FEBRUARY 26-MARCH 3, 1999 AMERICAN ACADEMY O, XP000916359 the whole document ---	1-4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2000	08.08.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	von Ballmoos, P

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/04780

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KIM, NACKSUNG ET AL: "Suppressive vaccination of allergen-induced immunoglobulin E production by the naked DNA vaccine." JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, (JAN., 1998) VOL. 101, NO. 1 PART 2, PP. S61. MEETING INFO.: 54TH ANNUAL MEETING OF THE AMERICAN ACADEMY OF ALLERGY, ASTHMA AND IMMUNOLOGY WASHINGTON, DC, USA MARCH 13-18, 1998 AMERICAN ACADEMY OF ALLERGY, , XP000916354 the whole document ---	1-4
X	HSU C H ET AL: "Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization 'see comments!." NATURE MEDICINE, (1996 MAY) 2 (5) 540-4. , XP000915972 cited in the application abstract page 541, left-hand column ---	1-3
X	RAZ E ET AL: "PREFERENTIAL INDUCTION OF A TH1 IMMUNE RESPONSE AND INHIBITION OF SPECIFIC IGE ANTIBODY FORMATION BY PLASMID DNA IMMUNIZATION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, 1 May 1996 (1996-05-01), pages 5141-5145, XP000652250 ISSN: 0027-8424 cited in the application abstract ---	1,2
A	WO 94 24281 A (IMMULOGIC PHARMA CORP) 27 October 1994 (1994-10-27) the whole document -----	1-4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/04780

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 2 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/04780

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9424281 A	27-10-1994	ZA 9302677 A	15-11-1993
		AU 680820 B	14-08-1997
		AU 4102693 A	08-11-1994
		EP 0694067 A	31-01-1996
		FI 954895 A	13-10-1995
		FI 963331 A	27-08-1996
		JP 9501043 T	04-02-1997
		NO 954095 A	13-12-1995